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E. FARKAS, J. HORVÁTH, S. KOTLÁN, R. MANNINGER,
A. PELC, K. RAUSS, J. SZIRMAI, J. WEISSFEILER

REDIGIT

G. IVÁNOVICS

TOMUS X

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IMMUNOFLUORESCENCE AND PASSIVE HAEMAGGLUTINATION IN INFANTILE ENTEROCOLITIS

By

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(Received August 31, 1962)

Summary. In order to detect *Shigella* excretors, 252 hospitalized children, one to fourteen years of age, have been examined. The examination of faeces and rectal samples was performed by cultural, immunofluorescent and passive haemagglutination methods.

Shigellae were cultured in 40.5 per cent from rectal samples taken on the day of admission, but only in 12.7 per cent from faeces obtained next day. Cultural methods were compared with the immunofluorescent method in 187 cases. By the latter, *Shigellae* were detected three times more frequently than by culturing. The combination of the three methods gave positive results in 50.6 per cent. The rise in the passive haemagglutination titre in 18 paired sera confirmed the positive results of the former methods. Rising titres were observed also in some patients who, although excreted bloody and mucous stools, yielded no evidence of *Shigella* infection by any other examination methods.

A considerable part of the cases of children's enterocolitis is due in Hungary to *Shigellae*. The causative agent frequently remains undetectable by routine bacteriological examination, and thus the patient is often discharged from hospital without a verified diagnosis. In order to reduce nosocomial infections [1] it is important to detect *Shigella* excretors and separate them from patients with other enteric diseases as soon and in as large numbers as possible. In the present studies the usual bacteriological examination of faecal samples was supplemented with a method promising more positive results than the others. In addition, on the basis of antibody titres, we have attempted to show that cases of enterocolitis with negative bacteriological findings were caused by *Shigellae* or by other pathogenic bacteria.

Materials and methods

Bacteriological examination. At admission, the patients were sampled rectally with Loeffler tubes as described by TEVELY [2]. The samples were immediately inoculated onto desoxycholate citrate agar plates. The plates were placed in incubators, which the hospital units had been provided with. Faecal samples were streaked similarly on desoxycholate citrate medium. Incubation lasted for 24 hours.

Immunofluorescent staining. The fluorescent tracer technique of COONS and KAPLAN [3] was used. *Shigella flexneri* and *sonnei* immune sera were labelled with Lissamine Rhodamine B-200 fluorescent dye, by the somewhat modified method of CHADWICK *et al.* [4, 5]. The dye was dissolved in acetone to make a saturated solution. Then the solution was added drop by drop to the buffered immune serum (pH 9) at 2° C until the pH was adjusted to 7.5 (approximately 1/2 hour). The conjugate was stirred with a magnetic mixer in the refrigerator overnight, then dialysed against saline for 4 days.

Smears. The faecal samples were suspended in five-fold amounts of saline, and the suspensions were left to stand for 10 minutes. Then the supernatants were smeared on defatted slides. The smears were fixed by heat and stained with the direct method.

Reading of smears. The smears were examined by means of the Leitz fluorescent microscope under blue light at magnifications of $400\times$ (dry) or $900\times$ (immersion).

Serum absorption. Immune sera were absorbed by *E. coli* strains. A 24 hour *E. coli* culture was centrifuged and the sediment resuspended in labelled Shigella immune sera. The suspension was incubated at 37°C for 1 hour, then the serum was separated by centrifugation. By this procedure the non-specific fluorescent reaction was mostly eliminated and the serum was freed from unbound dye.

Detection of antibodies. Antibodies were titrated by passive haemagglutination using the method of RAUSS and KÉTYI [6]. The serum samples were taken from each patient at admission and 14 days later.

Results

Table I shows a comparison of cultural examinations performed with rectal samples taken at admission and with faeces excreted next morning. Of 252 patients with clinical dysentery, the faecal samples were positive in 32 cases (12.6 per cent), the rectal samples in 102 cases (40.5 per cent).

Table I
Comparison of rectal and faecal cultures

R+ F+	R+ F-	R- F+	R- F-	Total No. of cases	F+	R+
25 (9.9%)	77 (30.6%)	7 (2.8%)	143 (56.7%)	252 (100.0%)	32 (12.7%)	102 (40.5%)

F = faeces

R = rectal sample

Table II
*Comparison of the results obtained with direct
fluorescent staining and faecal cultures*

Shigella	Fl+	B+	Total+	Fl-	B-	Total
Number	37	11	48	150	176	187
Per cent	19	5.8	25.6	80.2	94.2	100.0

Fl = fluorescent examination

B = bacteriological examination

With fluorescent staining the faeces of 75 patients were examined on alternative days throughout the course of the disease. Altogether 187 examinations were carried out with labelled *Sh. flexneri* and *sonnei* antisera.

Of the 187 examinations 37 (19 per cent) yielded positive results with fluorescent tracing; the faecal cultures were positive for shigellae in 14 cases (5.8 per cent) only. With the combination of the two methods 48 cases (25.6 per cent) were diagnosed.

These examinations showed that during the observation period the percentage of positive results decreased from 30 to 6. The number of stainable bacteria was probably reduced by the specific therapy. The lowest proportion of positivity was found on the 5th day after admission following a 4-day course of chloramphenicol. Later the proportion of positive results increased, as two patients remained *Shigella* excretors.

Table III

Positive results with fluorescent technique during the observation period

Days	No. of patients	Positive findings	
		No.	%
1	74	22	30
3	60	10	17
5	35	2	6
7	11	1	9
14	7	2	30

The combination of the 3 methods yielded positive results in 50.5 per cent at admission. Fluorescent staining and rectal cultures seemed to be equal in value, as both were positive in approximately 30 per cent. Faecal cultures yielded positive results with less than half of the frequency (12 per cent). However, in 4 cases (5 per cent) the causative agent was detected only at the routine faecal examination.

Table IV

*Comparison of faecal and rectal cultures
and fluorescent examination carried out at admission*

							No. of cases		
							Total	Positive	Negative
Faecal culture	+	+	+	-	-	-	75 (100.0%)	38 (50.6%)	37 (49.4%)
Rectal culture	+	+	-	+	+	-			
Fluorescent staining	+	-	-	+	-	+			
No. of cases	4	1	4	7	11	11			

The paired sera of 18 patients were examined. With these examinations we attempted to control and supplement the cultural and immunofluorescent methods.

Table V
Comparison of positive results obtained by the three methods

	Number of cases		Total
	Positive	Negative	
Faecal culture	9 (12%)	66 (88%)	75
Rectal culture	23 (30.6%)	52 (69.4%)	75
Fluorescent staining . . .	22 (29.3%)	53 (70.7%)	75

Table VI
*Comparison of faecal and rectal cultures,
 fluorescent tracing and serological examination*

	Faecal culture	Rectal culture	Fluorescent tracing	Serological examination
+	3 (16.6%)	4 (22.2%)	6 (33.3%)	14 (77.8%)
—	15 (83.4%)	14 (77.8%)	12 (66.7%)	4 (22.2%)
Total	18	18	18	18

The reciprocals of the passive haemagglutination titres were between zero and 256. The results confirmed the positive tests obtained with the 3 other methods.

Of 4 patients who were negative by all the three diagnostic tests, 3 gave antibody titres conclusive of *Sh. sonnei* and 1 of *Sh. flexneri* infection. The antibody titre remained unaltered during the observation period in 4 of the patients.

In most cases *Shigella* infection was indicated by the passive haemagglutination test (77.8 per cent).

Shigella infection was verified in 6 cases (33.3 per cent) by the fluorescent method, as compared to the 3 cases positive by faecal culture (16.6 per cent).

Discussion

The results of the present examinations have shown that rectal sample cultures and the fluorescent antibody technique are advantageous in the diagnosis of enterocolitis.

With faecal cultures the results were available only 60 to 72 hours after admission. The immediate inoculation of rectal samples shortened this period to 36 to 48 hours. In addition, the method yielded considerably more positive

results, probably because the culture medium had been seeded before drugs were administered. The technique of inoculation is simple and may be easily performed by the nurse.

The fluorescent staining method is the first procedure that allows within 1—2 hours the bacteriological diagnosis of an infection caused by a morphologically non-characteristic organism. In addition to its rapidity, the fluorescent method is less influenced by previous therapy. The diagnosis by fluorescent staining may be somewhat disturbed by an aspecific fluorescence due to serologically related bacteria. According to a model experiment, aspecific fluorescence may occur in approximately 1 per cent. Unfrequently, fluorescent tracing is negative with faecal samples giving positive cultural results.

In 1958 WHITAKER [7] used the immunofluorescent method for the rapid detection of excretors in a community of infants during an outbreak of *E. coli* infection. Later the method was used for the rapid tracing of a variety of micro-organisms [8—11]. Most authors reported on its use for the identification of pathogenic *E. coli* strains [12—15]. We have introduced the method in order to increase the number of results positive for Shigellae.

In the available literature no reports have been found on the fluorescent tracing of shigellae in human material. LABREC *et al.* [16] used the method for the examination of faecal samples of artificially infected guinea pigs.

We have elaborated a modification of the labelling of immune sera, by adding CHADWICK's Rhodamine B-200 to the immune sera so that the reaction of the serum is adjusted to pH 7.5.

By use of sera prepared by the modified conjugation technique, of the culturally positive faecal samples only one was negative by fluorescent staining. Out of the 47 samples 17 (34 per cent) were positive on culturing.

In contrast, out of 126 examinations performed with a less perfectly bound dye, 6 (4.7 per cent) were negative for shigellae and the total number of positive findings was only 15 (12 per cent). The result of fluorescent staining corresponded in 89.4 per cent to that of the cultural method used for the examination of the same faecal material. This is in accordance with the finding of COHEN *et al.* [14], who, with regard to *E. coli* excretion, reported this proportion as 87 per cent.

A considerable number of cases occurred when only the fluorescent method indicated Shigella infection. This may be due to the fact that fluorescence may be exhibited also by bacteria that had been killed as a result of therapy. Of the 33 cases found positive only by the fluorescent method in the hospital, 23 had been positive at outside examinations before admission. The negative cultural examinations within the hospital might have been due to the administration of drugs. It should be noted that certain *E. coli* strains are serologically related to Shigellae; however, these relationships might cause only negligible experimental errors.

During the observation period two *Shigella* excretors were found to be positive 14 days after the beginning of the usual therapy. One of them was culturally positive for *Sh. sonnei* at admission. Later, however, the causative agent was detected only by the fluorescent technique. In 40 per cent of the patients neither of diagnostic methods revealed *Shigellae*. Nevertheless, the change occurring in the antibody titre indicated the causative role of these bacteria.

It has been observed that in typical cases marked by mucus and blood containing stools, a rise in antibody titre invariably occurred. In contrast, in children excreting only diarrhoeal or mucous stools, the *shigella* aetiology could not always be verified. Of these patients those not harbouring *Shigellae* should be separated from the others, and only the true *Shigella* excretors are to be admitted to the dysentery department.

Because of the advantages described, for the examination of such patients the fluorescent method is the method of choice. When the examination is intended to include a search for more than one pathogenic organism, direct or indirect staining of the smears should be performed with polyvalent sera. The elaboration of a technique for this purpose is in progress.

The great advantage of the fluorescent staining method in infantile enterocolitis consists of the rapid detection of the causative agent and thus the prevention of introducing other kinds of pathogenic bacteria to the special units of enteric diseases.

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OLIGOSACCHARIDE DECOMPOSITION BY *CANDIDA SOLANI*

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(Received September 26, 1962)

Summary. It has been shown that the decomposition of sucrose to glucose and fructose by *Candida solani* is an intracellular process which is due neither to invertase nor to maltase (α -glucosidase). Thus *C. solani* does not contain these enzymes. The permeation of maltose is enhanced by an aerobic and an anaerobic permease. The anaerobic maltose permease is an adaptive enzyme.

Of the physiological and biochemical properties of yeasts the fermentation and assimilation of different carbohydrates are considered as the most important characteristics for the systematic classification of these organisms [1—3]. From data obtained in previous investigations and from theoretical conclusions the first steps of the fermentation and assimilation processes have been considered [4, 5]. In this paper experiments are described on the oligosaccharide fermentation of *C. solani*. This species ferments glucose and sucrose, and assimilates glucose, galactose, sucrose and maltose [1, 3]. According to our concept, such a behaviour suggests that the decomposition of sucrose occurs intracellularly by α -glucosidase, sucrose phosphorylase or some other enzyme, but undoubtedly not by invertase; the decomposition of maltose takes place also intracellularly by α -glucosidase, maltose phosphorylase or perhaps amylomaltase [4, 5].

Materials and methods

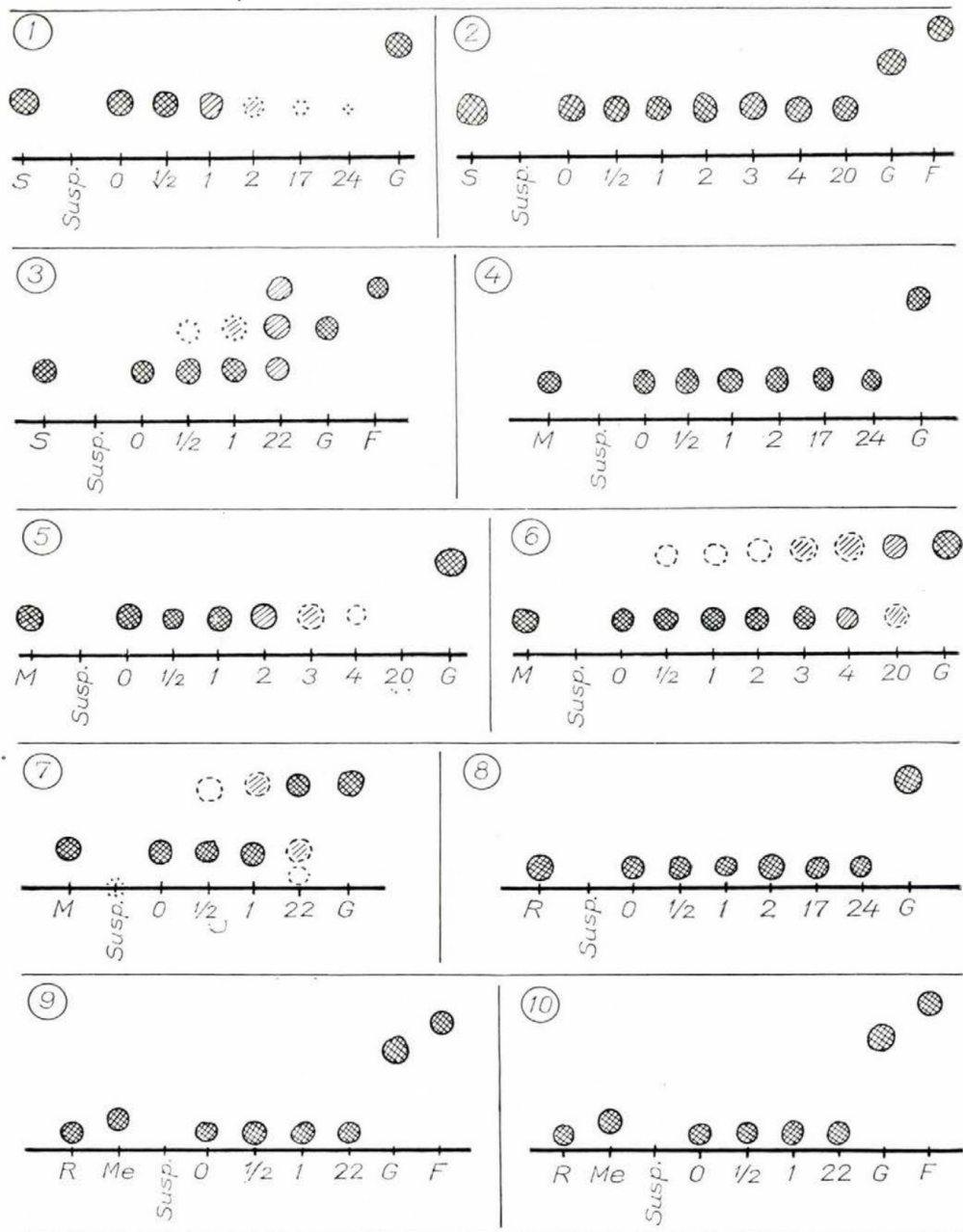
The *C. solani* strain used in this study has been maintained in our culture collection (*Department of Mycology*). Cultivation of the organism and other methods have been described previously [6—8].

For the fermentation experiments sucrose, maltose and raffinose were dissolved in isotonic saline or phosphate buffer (M/15, pH 7.2) in amounts indicated previously [7]. The chromatographic methods have also been described [6, 7].

Results

Cleavage of sucrose. Sucrose was taken up by living cells without extracellular splitting (Fig. 1). No action on sucrose was observed by acetone-treated cells (Fig. 2). The cell-free filtrate of homogenized cells converted sucrose to glucose and fructose (Fig. 3).

Cleavage of maltose. Maltose was not taken up by living cells (Fig. 4), although by pre-cultivating in maltose containing medium the strain could be adapted to anaerobic maltose uptake (Fig. 5). The adapted variant ferm-



ented this sugar. Both acetone-treated cells (Figs. 6) and the cell-free filtrate of homogenized cells (Fig. 7) produced glucose from maltose.

Cleavage of raffinose. Raffinose was attacked neither by living nor by acetone-treated and homogenized cells (Figs. 8—10). Living cells did not take up this substance (Fig. 8).

Discussion

It is clear from the chromatograms that the cleavage of sucrose is not due to invertase (β -h-fructosidase), which is an exo-enzyme in yeasts [6—9]. On the other hand, raffinose was uniformly left unattacked by living, acetone-treated or homogenized cells. Consequently the hydrolysis of sucrose occurs intracellularly, without any connection to the cleavage of maltose. This is confirmed by the finding that acetone-treated cells decomposed maltose but not sucrose. Thus, although it is known that maltase acts also on sucrose [10, 11], the common role of α -glucosidase in the splitting of sucrose and maltose can be rejected.

Fig. 1. Sucrose decomposition by living *C. solani*. Isotonic NaCl, living cells, 100 mg wet weight; 10 mg sucrose. Left: sucrose and suspension controls. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 2. Sucrose decomposition by acetone-treated *C. solani*. *M/30*, pH 7.2 phosphate buffer; acetone-treated cells, 400 mg live wet weight; 10 mg sucrose. Left: sucrose and suspension controls. Right: glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

Fig. 3. Sucrose decomposition by the filtrate of homogenized *C. solani* cells. *M/30*, pH 7.2 phosphate buffer; filtrate of homogenized cells, 400 mg live wet weight; 10 mg sucrose. Left: sucrose and suspension (filtrate) controls. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 4. Maltose decomposition by living *C. solani*. Isotonic NaCl, living cells, 100 mg wet weight; 10 mg maltose. Left: maltose and suspension controls. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 5. Maltose decomposition by living *C. solani*. *M/30*, pH 7.2 phosphate buffer, living cells 400 mg wet weight; 10 mg maltose. Left: maltose and suspension control. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 6. Maltose decomposition by acetone-treated *C. solani*. *M/30*, pH 7.2 phosphate buffer; acetone-treated cells, 400 mg live wet weight; 10 mg maltose. Left: maltose and suspension control. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 7. Maltose decomposition by the filtrate of homogenized *C. solani* cells. *M/30*, pH 7.2 phosphate buffer; filtrate of homogenized cells, 400 mg live wet weight; 10 mg maltose. Left: maltose and suspension control. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 8. Raffinose decomposition by living *C. solani*. Isotonic NaCl, living cells 100 mg wet weight; 20 mg raffinose. Left: raffinose and suspension controls. Right: glucose control. Numbers on the start line mean the sampling intervals in hours

Fig. 9. Raffinose decomposition by acetone-treated *C. solani*. *M/30*, pH 7.2 phosphate buffer; acetone-treated cells 400 mg live wet weight; 20 mg raffinose. Left: raffinose, melibiose and suspension controls. Right: glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

Fig. 10. Raffinose decomposition by the filtrate of homogenized *C. solani* cells. *M/30*, pH 7.2 phosphate buffer; filtrate of homogenized cells, 400 mg live wet weight; 20 mg raffinose. Left: raffinose, melibiose and suspension controls. Right: glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

The cleavage of maltose occurs intracellularly and the necessary enzyme is contained in the cell (Fig. 6 and 7). Under anaerobic conditions maltose does not permeate, but the cells may be adapted to the anaerobic uptake and fermentation of this sugar. For this process a separate maltose permease (maltose transportase) enzyme, different from the aerobic maltose permease, is responsible. One constituent of the polymer maltose fermentation gene (genes) suggested by WINGE and ROBERTS [12] may be the factor that controls maltose permeases. The role of α -glucosidase (maltase) in the cleavage of maltose is not probable in this case, because, as it has already been referred to, this enzyme acts also on sucrose. Thus acetone-treated preparations would act on both of these disaccharides. It seems improbable that after acetone treatment only maltose, while before acetone treatment only sucrose, should reach the α -glucosidase localized somewhere in the cell, and in non-acetonized cells maltose would not permeate at all. This would mean that acetone treatment lifts the permeation block before maltose and at the same time stops the permeation of sucrose.

Raffinose is not split by living, acetone-treated or homogenized *C. solani* cells. Living cells do not take up this substance. As mentioned above, the very lack of action on raffinose shows that the cleavage of sucrose is not due to invertase.

Accordingly, our concept that yeasts are able to split sucrose in lack of invertase, that is, the fermentation of sucrose may take place without the cleavage of raffinose, has been confirmed. In that case the cleavage of sucrose is not necessarily taken over by α -glucosidase. Data regarding the taking over of sucrose decomposition by α -glucosidase were reported by KOSIKOV, GELMAN and RAEVSKAJA [10].

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BIOCHEMICAL REACTIONS AND VIRULENCE OF *E. COLI* O124: K72 (17)

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Summary. Although some of the biochemical reactions of *E. coli* O124 and shigellae are similar, O124 strains belong biochemically to the late lactose-fermenting group of *E. coli*. At oblique illumination various colonial variants can be distinguished in *E. coli* O124 cultures. The variants may differ in virulence. In guinea pigs infected conjunctivally with virulent *E. coli* O124 cultures, typical keratoconjunctivitis can be produced. As to natural, acquired immunity, *Shigella* and *E. coli* O124 strains can be mutually substituted and a cross resistance can be produced.

In addition to shigellae, *E. coli* O124 is a well defined group of bacteria capable of causing experimental keratoconjunctivitis in guinea pigs [11, 16, 20]. The purpose of the present investigation was to gain further knowledge of the common principle determining the pathogenicity of both shigellae and *E. coli* O124.

Materials and methods

Biochemical reactions were performed by generally accepted methods [2, 6]. Decarboxylase tests were carried out by CARLQUIST's method [1] and MØLLER's method [8, 9], as modified by HORMAECHE and PELUFFO [4]. Biochemical reactions were evaluated by the schema of KAUFFMANN, EDWARDS and EWING [7]. The reactions were read after 4 minutes with CARLQUIST's, and after 4 days with SIMMONS' and MØLLER's, tests.

The technique of conjunctival infection and oblique illumination at different angles have been described previously [13—18]. Most of the colonial variants were obtained by incubating the original strain in U tubes containing 0.2 per cent agar. When the culture appeared in the other arm of the U tube, it was streaked on yeast extract agar plates [19], then the variant colony was subcultured serially.

Results

Biochemical reactions. The biochemical reactions of 50 *E. coli* O124 strains are summarized in Table I. The strains corresponded to late lactose fermenting, salicin negative, *E. coli*. The motility of the cultures was weak, they took 2 to 10 days to pass through the U tubes.

Parallel examination of different *Shigella* and *E. coli* types was performed in order to determine the salicin and sucrose reaction and growth in SIMMONS' liquid ammonium glucose medium [22]. As shown in Table II, *E. coli* O124 strains are similar to shigellae in the fermentation of salicin or sucrose; however,

they can utilize glucose and ammonium salts as C or N sources almost as well as the members of other *E. coli* groups.

Table I
Biochemical reactions of E. coli O124 strains

Adonitol	—	Lactose	(+)	Indole	+
Dulcitol	d	Sucrose	d	H ₂ S	—
Sorbitol	+	Mannitol	++	Gelatin	—
Arabinose	+	Glucose	++	V. P.	—
Xylose	+	Melibiose	+	M. R.	+
Rhamnose	d	Cellobiose	—	Urea	—
Maltose	+	Raffinose	x	KCN	—
Salicin	—	Trehalose	+		
Inositol	—	Ribose	+	Motility	(+)

— negative
+ positive
++ acid and gas

(+) late positivity
x late and irregularly positive or negative
d different biotypes

Table II
Biochemical reactions of shigellae and some E. coli strains

Strain	No. of strains	Salicin			Sucrose			Ammonium glucose (Simmons)		
		+	(+)	—	+	(+)	—	+	(+)	—
<i>Sh. dysenteriae</i> 1—4	11	—	—	11	—	—	11	—	—	11
<i>Sh. flexneri</i> 1,2,4—6	41	—	—	41	1	7	33	—	—	41
<i>Sh. flexneri</i> 3	15	—	—	15	2	12	1	14	1	—
<i>Sh. boydii</i> 1—15	17	—	—	17	—	2	15	—	—	17
<i>Sh. sonnei</i>	11	—	2	9	—	10	1	—	—	11
<i>E. coli</i> O1—O25	23	13	4	6	5	1	17	23	—	—
<i>E. coli</i> *	77	56	6	15	40	9	28	77	—	—
O124	100	—	1	99	—	8	92	80	20	—

* *E. coli* O26, O55, O86, O111, O119, O125, O126, O127, O128

It was remarkable that when tested as to the Carlquist reaction, O124 cultures behaved similarly to shigellae as far as they did not produce lysine decarboxylase (Table III). Therefore MØLLER's decarboxylase reactions were also carried out (Table IV). It was shown that, although after a prolonged time, the majority of O124 strains were able to decarboxylate lysine. No significant difference was revealed between O124 and other *E. coli* antigenic groups in

enzymic activity on other amino acids. Only the frequent absence of ornithine decarboxylase was noted.

Thus, between *E. coli* O124 and *Shigella* no such important biochemical similarity has been shown which would explain their related pathogenicity.

Table III
LDC reactions (CARLQUIST's method)

Strain	No. of strains	LDC	
		+	-
<i>Shigella</i>	18	—	18
<i>E. coli</i> O124.....	177	—	177
<i>E. coli</i> other groups	67	60	7
<i>Salmonella</i>	16	16	—

Table IV
DC reactions (Møller's method)

Strain	No. of strains	LDC			ADC			ODC			GDC ⁴		
		+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>Sh. dysenteriae</i> 1—4	11	—	—	11	—	2 ²	9	—	—	11	11	—	—
<i>Sh. flexneri</i> 1,2,3,5	30	—	—	30	—	—	30	—	—	30	30	—	—
<i>Sh. flexneri</i> 4	18	—	—	18	—	2	16	—	9	9	18	—	—
<i>Sh. flexneri</i> 6	8	—	5	3	5	3	—	—	2	6	8	—	—
<i>Sh. boydii</i> 1—15	17	—	1	16	7	7	3	2 ¹	—	15	11	1 ¹	5 ¹
<i>Sh. sonnei</i>	11	—	2	9	3	7	1	11	—	—	11	—	—
<i>E. coli</i> O1—O25	23	.	.	.	7	15	1	14	6	3	23	—	—
<i>E. coli</i> ³	64	57	7	—	7	57	—	40	24	—	64	—	—
O124	86	—	73	13	6	80	—	4	48	34	85	—	1 ¹

¹ laboratory strains

² one laboratory strain among them

³ *E. coli* O26, O55, O86, O111, O119, O125, O126, O127, O128

⁴ LDC = lysine decarboxylase, ADC = arginine decarboxylase, ODC = ornithine decarboxylase, GDC = glutamic acid decarboxylase

Colonial morphology and virulence. With oblique light at different angles in addition to turbid and highly turbid colonies recognizable in *E. coli* O124 cultures [6], very highly, highly, or moderately refractive colonies may be shown. The colonies may be homogeneous, or contain concentric or network-like formations [17, 21]. The network variant is always avirulent for the conjunctivally infected guinea pig.

No parallelism has been shown between mouse and guinea pig virulence (Table V). With experimental conjunctival infection the homogeneous variant was virulent, the concentric variant was weakly virulent and the network variant was avirulent for guinea pigs.

In their majority, the biochemical reactions of the different colonial variants were uniform. The discrepant results are summarized in Table VI.

Table V

Mouse virulence of the colonial variants of E. coli O124 strain 22/529

No. of infecting organisms	No. of animals killed in groups of 20 mice inoculated with		
	Homogeneous variant	Concentric variant	Network variant
5 ⁶	5	2	6
5 ⁷	10	10	10
5 ⁸	17	16	15
LD 50	5 ⁷	5 ⁷	5 ⁷

Table VI

Differences in the biochemical reactions of E. coli O124 colonial variants

Variant	Lactose	Rhamn- ose	Sucrose	Raffin- ose	Arginine	Ornith- ine	Motility
22/529							
Highly refr. homogeneous viru- lent	+3	+3	—	—	+2	—	+8
22/529							
Highly refr. concentric weakly virulent	+3	+2	—	—	+2	—	+8
22/529							
Network avirulent	+8	+3	—	+3	—	—	+8
620/7b							
Very highly refr. homogeneous virulent	—	+9	+4	+2	+1	—	+8
620/3b							
Very highly refr. concentric weakly virulent	+12	+24	—	—	+1	—	+8
620/1b							
Highly refr. homogeneous virulent	+12	+4	—	+6	+3	—	+5
620/2b							
Highly refr. concentric weakly virulent	+12	+7	—	—	+2	—	+8
620/6b							
Medium refr. homogeneous avirulent	—	+9	—	—	+2	+3	+5

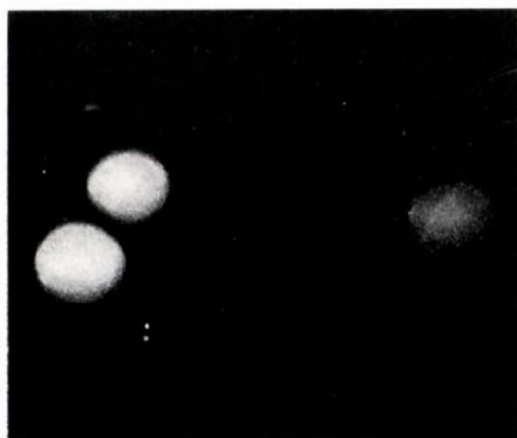


Fig. 1. Homogeneous, highly refractive O124 colonies. (On the right a weakly refractive homogeneous colony.) Magnification, approx. $3\times$



Fig. 2. Homogeneous, very highly refractive O124 colony. Magnification, approx. $3\times$

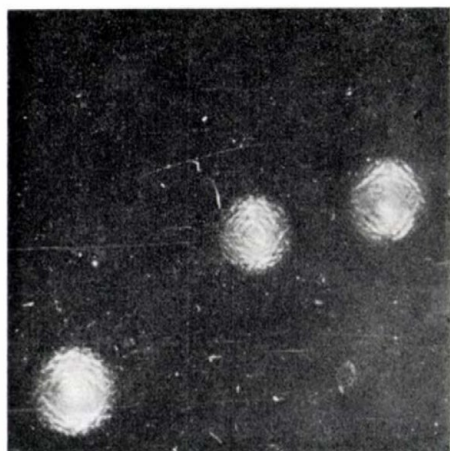


Fig. 3. Concentric, highly refractive O124 colonies. Magnification, approx. $3\times$. The filamentous formations are generally running parallel



Fig. 4. Concentric, very highly refractive O124 colony. Magnification, approx. $3\times$

It can be concluded from the results that no regular correlation exists between virulence and biochemical activity.

After conjunctival infection with virulent *E. coli* O124 variants, in guinea pigs keratoconjunctivitis develops, which, in clinical symptoms, course and, according to the examinations of Dr. P. RÁCZ, histologically, corresponds to *keratoconjunctivitis shigellosa*. The members of *E. coli* O antigenic group 124 are known to be pathogenic for man and often cause dysenteriform diseases.

Natural, acquired resistance. After recovery from keratoconjunctivitis due to *E. coli* O124, local tissue immunity develops, not only to the homologous organism, but also to various *Shigella* types. The alternative of this can also be demonstrated (Table VII).

Discussion

It has been shown that virulent strains of both *E. coli* O124 and *Shigella* produce keratoconjunctivitis in the guinea pig. After recovery from the infection, cross-immunity can be demonstrated.

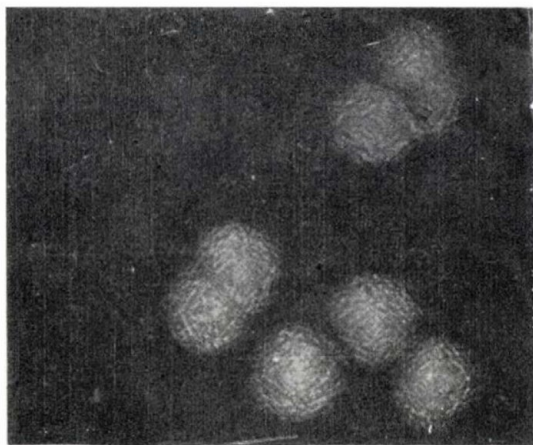


Fig. 5. Moderately refractive network colonies. Magnification, approx. $3\times$. The filamentous formations crossing each other produce a network

The common virulence factor presumably occurring in both groups of microorganisms could not be determined. It may be assumed that this factor cannot be detected with the usual biochemical reactions; it is recognizable only with serological methods. This seems well established by the fact that the somatic antigens of *E. coli* O124 and *Sh. dysenteriae* 3 are identical.

The cross-immunity between O124 and *Shigella* strains is interesting, as such observation regarding shigellae and serotypes 792 and 147 have already been observed [23]. In contrast, between *Shigella* and *Listeria* no cross-immunity exists [24]. The findings indicate that the "keratoconjunctival factors" of *Shigella* and *E. coli* O124 on the one hand, and that of *Shigella* and serotypes 792 and 147 on the other, are identical.

Within the family Enterobacteriaceae the arbitrarily demarcated groups cannot be separated sharply. The demarcation line should be drawn at the periphery of dense population centres [3]. The *Shigella* and *Escherichia* groups are related both serologically and biochemically. Accordingly, it cannot be

Table VII
Cross-immunity between Shigella and E. coli O124 infection

No.	Culture used for first infection	Culture used for second infection	Immunity
1.	<i>Sh. flexneri</i> 1b	<i>E. coli</i> O124	+++
2.	1b	"	+++
3.	2a	"	+++
4.	3	"	+++
5.	<i>E. coli</i> O124	<i>Sh. flexneri</i> 2a	+
6.	"	2a	+
7.	"	2a	++
8.	"	2a	+++
9.	"	3	—
10.	"	3	—
11.	"	3	—
12.	"	3	+
13.	"	3	++
14.	"	<i>Sh. sonnei</i>	+
15.	"	"	++
16.	"	"	+++
17.	"	"	+++
18.	"	"	+++
19.	"	"	+++
20.	"	"	+++
21.	"	"	+++

regarded exceptional that the two groups should possess common features in virulence. Thus, certain *E. coli* groups and serotypes may give rise to pathological changes in the eye of the guinea pig (e.g. strain "Öskü" [12], serotypes 792 and 147 [23] and O124). Despite these considerations, we think that to continue to name the model disease as keratoconjunctivitis shigellosa is justified. Experimental keratoconjunctivitis can be constantly produced with any group or serotype of virulent *Shigella* strains. The *Shigella* strain virulent for guinea pigs produces dysentery in man [5], or, alternatively, cultures avirulent for guinea pigs are avirulent for man as well [11, 5]. That the name of the disease is correct is further confirmed by the identity of the somatic antigens of *E. coli* O124 and *Sh. dysenteriae* 3. When the guinea pig's eye can be infected

with *E. coli* strains, it is more advisable to use the term "*E. coli* keratoconjunctivitis".

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CORRELATION BETWEEN PHAGE-TYPE, COAGULASE, HYALURONIDASE AND PHOSPHATASE ACTIVITY, AND MERCURIC CHLORIDE RESISTANCE OF STAPHYLOCOCCUS AUREUS

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Summary. Phage group I *Staphylococcus aureus* strains (especially phage type 80/81) exert a low coagulase and hyaluronidase but high phosphatase activity. Strains belonging to phage group II show a high coagulase and hyaluronidase and a medium phosphatase activity. Phage group III strains produce small amounts of phosphatase; their hyaluronidase and coagulase activity is of a medium degree.

Most of the "epidemic" strains are resistant to mercuric chloride. The resistance is not associated with the increased production of any of the examined enzymes.

In order to determine their pathogenicity, the biological activity of staphylococcal strains has been widely examined. BURNS and HOLTMAN [1] have summarized the biochemical characters important for the differentiation of pathogenic staphylococci. The difference in the virulence of pathogenic strains was first established by use of WILLIAMS and RIPPON's phage typing method [2]. It has been shown that staphylococcal epidemics are caused most frequently by certain phage types, for example, in Hungary by type 80/81 [3].

A number of investigations have been carried out to reveal biochemical properties that may be used for distinguishing "epidemic" strains from other pathogenic staphylococci. VÁCZI *et al.* [4] and MOORE [5] found that most of the epidemic strains were 30 times more resistant to mercuric chloride than other pathogenic *Staph. aureus* cultures. Mercuric chloride resistance is also connected with antibiotic susceptibility. VÁCZI *et al.* [4] and AKINLADE [6] have shown that highly antibiotic resistant strains, too, are resistant to mercuric chloride. The association between hyaluronidase activity and phage type was shown by FABER and ROSENDAL [7]. PAN and BLUMENTHAL described the correlation of acid phosphatase activity and phage type [8]. This paper presents a comparison of mercuric chloride resistance and antibiotic sensitivity with the quantitatively estimated hyaluronidase, phosphatase and coagulase activity in staphylococci of various phage groups.

Materials and methods

Cultures. Pathogenic *Staph. aureus* strains used in this study were isolated and phage typed by the method of WILLIAMS and RIPPON [2] in the Laboratory of Phage Typing, State Institute of Hygiene, Budapest. All strains produced coagulase, split mannitol, liquefied gelatin and formed pigment.

Quantitative coagulase assay. Into each of the hollows in TAKÁTSY's plastic plates, 0.05 ml citrated human plasma was dropped by means of TAKÁTSY's pipette [9]. The plasma was prepared by centrifuging the mixture of 1 part 3.8 per cent sodium citrate and 4 parts of blood.

Serial dilutions of 20 hour broth cultures were performed with the 0.05 ml spiral loop. The plates then were incubated at 37° C for 2 hours. Readings were made at transmitted light provided by a 50 W opal bulb.

For each strain 2 parallel dilution series were prepared. The titre is given as the reciprocal of the highest dilution of the broth culture causing coagulation. The experiments were repeated three times and the mean titres were calculated.

Quantitative hyaluronidase assay was performed by ÚJVÁRY's method [10], with ox synovia as the substrate. The reaction mixture contained 1.2 ml 1 : 4 diluted synovia and 0.6 ml 20 hour broth culture and its dilutions. Incubation lasted for $\frac{1}{2}$ hour in a 37° C water bath, then to each tube 6 ml reagent was added. The reagent consisted of 1 part 2 N acetic acid and 9 parts 96 per cent ethanol. The titre was expressed as the reciprocal of the highest dilution causing the complete decomposition of hyaluronic acid. As a control hyaluronidase (Hyason, Organon, Oss) was used. In our system, 0.8 μ g of that preparation was necessary for the complete decomposition of hyaluronic acid.

Quantitative determination of phosphatase was carried out by the somewhat modified method described by BARNES and MORRIS [11]. The reaction mixture contained 3.8 ml sodium acetate — acetic acid buffer (0.1 N, pH 5.6), 0.2 ml 0.5 per cent p-nitro-phenyl phosphate disodium (Sigma) and 1.0 ml broth culture. The culture was grown at 37° C for 20 hours. The reaction mixture was incubated in a 37° C water bath for 30 minutes. Then 1 ml N NaOH was added to each tube to stop the reaction. After centrifugation the optical density of the supernatant was measured against the control solution in an Uvifot photometer at 405 m μ , in a 5 ml cuvette. The control solution contained sterile broth in place of the culture suspension, and was treated similarly as the reaction tubes. A standard curve was obtained with p-nitrophenol. The amount of p-nitrophenol liberated by 1 mg dry bacteria was expressed in micrograms.

Broth cultures. In every experiment broth cultures incubated for 20 hours at 37° C were used. The broth contained 1 per cent beef extract, 1 per cent peptone and 0.5 per cent NaCl. The pH was adjusted to 7.6 with N NaOH.

The cell count of the cultures was determined photometrically, using a standard curve. All experimental results concern 350 million cells/ml cultures.

Antibiotic sensitivity was estimated by the method of LÁSZLÓ *et al.* [12]. Agar plates were seeded with 1 ml aliquots of 1 : 10 diluted 20 hour broth cultures. The plates were left to stand at room temperature for 90 minutes, then the discs were placed. The discs, 5 mm in diameter, were prepared from Schleicher—Schuell No. 2043/B paper and contained 50 μ g of penicillin, streptomycin, terramycin or aureomycin, and 30 μ g of chloramphenicol or erythromycin. Strains producing inhibition zones larger than 25 mm were regarded sensitive, those with inhibition zones smaller than 8 mm were regarded resistant.

Mercuric chloride sensitivity was estimated by the disc method of VÁCZI *et al.* [4]. Schleicher—Schuell No. 2043/B filter paper strips were impregnated with 1 per cent mercuric chloride solution. After drying, discs 5 mm in diameter were cut. The examined strain was regarded sensitive when the inhibition zone was larger than 8 mm, and resistant when it was smaller than 8 mm.

Results

The coagulase, acid phosphatase and hyaluronidase activity and mercuric chloride and antibiotic sensitivity of 115 pathogenic *Staph. aureus* strains was determined. When the results are arranged according to phage groups, it is seen that phage group I strains produced small amounts of coagulase and hyaluronidase and high amounts of phosphatase. Most of these strains were resistant to mercuric chloride. As to antibiotic sensitivity, their majority fell into a group named "P. resistant"; the members of this group were resistant to penicillin, but sensitive to streptomycin, chloromycetin, terramycin, aureomycin and erythromycin (Table I).

Table I

Quantitative enzymic activity, mercuric chloride and antibiotic sensitivity of Staph. aureus cultures belonging to different phage groups

Phage group	No. of strains	Mean coagulase titre	Mean hyaluronidase titre	Phosphatase μg p-nitro-phenol/mg bacteria	HgCl ₂ resistance %	Antibiotic sensitivity %		
						S	P. res.	R
I	40	7.9	11.4	296	92.9	2.3	93.1	4.6
II	17	24.7	45.8	145	—	23.5	70.5	5.8
III	25	15.3	23.6	91.7	52	8	36	56
Untypable	8	13.7	29.5	131.7	87.5	12.5	37.5	50
Mixed	11	14.1	36.3	245.1	81.9	18.2	72.7	9.1
187	7	14.2	45.7	105	14.2	42.8	42.8	14.3
756/950	7	10.3	57.1	119.2	42.8	—	28.6	71.4

S: Sensitive to penicillin, streptomycin, chloromycetin, terramycin, aureomycin and erythromycin

P. res.: Resistant to penicillin, sensitive to streptomycin, chloromycetin, terramycin, aureomycin and erythromycin

R: Resistant to three or more of the above antibiotics

Phage group II strains produced large amounts of hyaluronidase and coagulase, but their phosphatase activity was low. They were sensitive to mercuric chloride and belonged mostly to antibiotic sensitivity group "P. resistant". A number of the strains was sensitive also to penicillin, so these were included in group "Sensitive".

The phosphatase activity of phage group III strains was very low. They produced medium amounts of coagulase and hyaluronidase. Of the strains 52 per cent was resistant to mercuric chloride. The strains were generally resistant to more than one antibiotic (group "Resistant") or belonged to group "P. resistant".

Untypable staphylococci produced medium amounts of coagulase, hyaluronidase and phosphatase; most of them belonged to sensitivity group "Resistant" and generally exhibited mercuric chloride resistance. No conclusions can be drawn from the results regarding the comparatively small number of other types. However, the high hyaluronidase activity and mercuric chloride sensitivity of type 187 is noteworthy. A similar high hyaluronidase activity was shown by type 756/950, which was mostly resistant to more than one antibiotic.

Table II shows the results for phage group I strains. Within phage group I the high phosphatase and low coagulase and hyaluronidase activity of 80/81 and 52/52A/80/81 types should be noted. The strains were resistant to mercuric chloride almost in 100 per cent, and belonged mainly to sensitivity group "P. resistant".

Table II

Quantitative enzymic activity, mercuric chloride and antibiotic sensitivity of Staph. aureus cultures belonging to phage group I

Phage type	No. of strains	Mean coagulase titre	Mean hyaluronidase titre	Phosphatase μg p-nitro-phenol/mg bacteria	HgCl ₂ resistance %	Antibiotic sensitivity %		
						S	P. res.	R
80/81	19	6.2	7.7	344.4	94.7	5.3	89.5	5.3
52/52A/80/81	6	4.8	8	311	83.4	—	83.4	16.6
79	9	12	16.4	195.9	88.9	—	100	—
29	8	10.4	17	283.3	100	—	100	—

The coagulase and hyaluronidase activity of phage type 79 strains was higher than that of the former types, but lower than the mean titre obtained with other types. As to phosphatase activity, the strains fell between 80/81 or 52/52A/80/81 and the other types.

Phage type 29 was similar in coagulase and hyaluronidase activity to type 79. As to phosphatase, it was more active than the latter, as it reached the level produced by type 80/81. The strains were resistant to mercuric chloride and belonged to sensitivity group "P. resistant".

Part of the strains was isolated from hospital epidemics. The enzymic activity of these strains was far from uniform (Table III). Low coagulase activ-

Table III

Quantitative enzymic activity, mercuric chloride and antibiotic sensitivity of Staph. aureus phage types isolated from hospital outbreaks

Phage type	No. of strains	Mean coagulase titre	Mean hyaluronidase titre	Phosphatase μg p-nitro-phenol/mg bacteria	HgCl ₂ resistance %	Antibiotic sensitivity %		
						S	P. res.	R
80/81	9	4.8	8.8	386.3	100	—	100	—
52/52A/80/81	5	4.5	8	302.7	80	—	80	20
79	8	13.5	8.5	161.4	100	—	100	—
6/47/54	9	16.8	6.6	81.6	88.9	—	—	100
75/77	7	15.3	25.7	132.6	57.2	28.6	28.6	42.9
756/950	4	7	72	83.4	50	—	25	75

ity was excreted by types 80/81, 52/52A/80/81 and 756/950. A medium amount of coagulase was produced by types 79, 6/47/54 and 75/77. With the exception of types 75/77 and 756/950, hyaluronidase production was low. Phosphatase activity was very high in 80/81 and 52/52A/80/81 strains, medium in 79 and

75/77 strains and low in 6/47/54 and 756/950 strains. Most of the epidemic strains belonged to the antibiotic sensitivity groups "P. resistant" and "Resistant". The correlation was the most uniform with mercuric chloride sensitivity: types 756/950 and 57/77 were resistant to this substance in 50 and 57 per cent, respectively, while other types were resistant in 80 to 100 per cent.

It was assumed that strains of the same phage type exhibiting different mercuric chloride resistance might be different in virulence. Table IV shows

Table IV

Quantitative enzymic activity and antibiotic sensitivity of different Staph. aureus phage groups according to mercuric chloride sensitivity

Phage group	No. of strains	Mean coagulase titre	Mean hyaluronidase titre	Phosphatase μg p-nitro-phenol/mg bacteria	Antibiotic sensitivity %		
					S	P. res.	R
I. HgCl ₂ res.	39	8	9.8	296	—	94.9	5.1
HgCl ₂ sens.	3	7	32	296	33.3	66.6	—
II. HgCl ₂ res.	—	—	—	—	—	—	—
HgCl ₂ sens.	17	24.7	45.8	145	23.5	70.5	5.8
III. HgCl ₂ res.	13	16.0	14.4	75.6	—	7.7	92.3
HgCl ₂ sens.	12	14.6	33.6	109.3	16.6	66.6	16.6

that mercuric chloride sensitive and resistant strains did not differ significantly in coagulase activity. Mercuric chloride sensitive strains of phage groups I and III produced more hyaluronidase than strains resistant to this substance. No difference was revealed in phosphatase activity. Strains sensitive to all antibiotics did not occur among mercuric chloride resistant cultures.

Discussion

Bacterial virulence is a resultant of the biological and biochemical properties. It may be assumed that the metabolism of virulent *Staph. aureus* strains differs qualitatively and quantitatively from that of other pathogenic *Staph. aureus* strains. The coagulase and hyaluronidase activities examined in the present study are important indicators of pathogenicity [13—18]. In addition, acid phosphatase activity and mercuric chloride resistance have also been examined.

It has been shown that *Staph. aureus* 80/81, which is regarded as the most widely spread epidemic phage type, has a characteristically low coagulase and hyaluronidase and high phosphatase activity. The other strains of phage group

mostly behaved similarly (Table I). The same results were obtained by FABER and ROSENDAL [7] for hyaluronidase and by PAN and BLUMENTHAL [8] for phosphatase activity.

INNIS and SANCLEMENTE [19] found that even chromatographically purified coagulase retained some phosphatase activity. From experiments carried out with inhibitors selectively paralyzing enzymic activity, these authors concluded that on the same protein carrier two different active groups were responsible for coagulase and phosphatase activity. It may be supposed that the two kinds of enzymic activities are connected. We have shown that the highest amount of phosphatase was generally produced by phage type 80/81 strains exerting a low coagulase activity. In phage group III strain 58, which belonged to phage type 47/53 and produced medium amounts of coagulase, completely lacked phosphatase activity. These data are indicative of a basic difference in the production of the two enzymes.

Our investigations have shown that the degree of coagulase activity is not correlated with the degree of virulence, since phage type 80/81 staphylococci, known as the most virulent strains, produce much less coagulase than the less virulent strains in phage group II (Table I).

Phosphatase activity is high in phage group I, to which the most virulent organisms belong. This property, however, cannot be regarded as the sole indicator of virulence, as virulent strains isolated from nosocomial epidemics, which fell into other phage types, exerted a very low phosphatase activity (Table III).

The finding of GILISSEN and RUDA [20], that penicillin resistant strains produce increased amounts of phosphatase, was not confirmed by the present experiments, as penicillin resistant strains belonging mainly to phage group, III had the lowest phosphatase activity among strains of any phage group (Table I).

Although no correlation was revealed between hyaluronidase production and virulence, it should be mentioned that, similarly to coagulase production the highest amounts of hyaluronidase were formed by phage group II strains that usually caused sporadic infections.

Accordingly although coagulase and hyaluronidase production is a property of pathogenic staphylococci, the increased production of these enzymes cannot be associated with an increased virulence. Virulent strains may be characterized by a high phosphatase and low coagulase and hyaluronidase activity.

A comparison of the mercuric chloride resistance with the quantitatively estimated enzymic activity has shown that mercuric chloride resistant and sensitive strains do not differ in coagulase and phosphatase production. Although the small number of mercuric chloride sensitive strains does not allow for definite conclusions, these cultures seem to have a higher hyaluronidase activity than resistant strains.

Staph. aureus strains responsible for outbreaks were, in accordance with the findings of VÁCZI *et al.* [4], mainly resistant to mercuric chloride (Table III). This behaviour cannot be explained simply by their penicillin resistance, as a number of penicillin resistant cultures were sensitive to mercuric chloride (e.g. in phage group II, Table I).

The experiments have shown that virulence is a resultant of complex metabolic processes, and thus it cannot be characterized by the activity of one single enzyme system. Virulence is better indicated by the proportion of the enzymes responsible for the pathogenicity of the strain.

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ADAPTATION OF YEASTS TO DISINFECTANTS

By

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Summary. Two *Saccharomyces cerevisiae* strains used in alcoholic fermentation have been adapted to formalin, active chlorine, cetylpyridinium and sodium penta-chlor phenolate. Adaptation was started at low concentrations of the disinfectants. With adaptation to formalin a 3-fold, to chlorine a 10-fold, to cetylpyridinium a 3-fold, and to sodium penta-chlor phenolate a 5-fold increase in resistance was obtained. Control examinations showed that the adapted yeasts were able to ferment at disinfectant concentrations destroying the unadapted strains. In media containing disinfectants a prolonged lag phase was observed; the fermentation rate in the logarithmic phase, the final alcohol, and yeast production and residual sugar content, however, were similar to that found in the control medium.

In industrial alcohol and yeast production and other large scale fermentation procedures, continuous production is considered to be the ultimate aim. However, its introduction is considerably hindered by the danger of contamination, which often causes difficulties even in batch operation. Contamination may be avoided by sterilizing the utensils and media by heat or chemical agents. Alternatively, the contaminating bacteria may be inhibited by suitable concentrations of selective substances incorporated in the fermentation medium. The elimination of these organisms is made difficult by the fact that generally microorganisms similar in life conditions to the yeast are encountered, and that the antagonistic action of the yeast decreases when it is grown in pure culture for prolonged periods.

In industrial alcohol and yeast production mainly acids, chlorine, formalin and cetylpyridinium are used as disinfectants. Recently, sodium penta-chlor phenolate has been introduced [1, 2]. The bactericidal effect of the different disinfectants is variable; it shows a non-linear relationship to concentration and a specific efficiency curve [3]. Microorganisms vitiating alcoholic fermentation are chiefly acid-producing bacteria, which, on the one hand cause a loss in nutrient substances by using them for metabolic processes. On the other hand, the produced acids lower the pH of the mash considerably, and thus, together with other metabolic products, inhibit or even stop the growth of the yeast and cause deficiencies in the quality of the product [4].

The purpose of the present experiments was to increase the selectivity of various disinfectants by applying them at concentrations inhibiting the growth of bacteria, but not influencing the fermentative activity of yeasts.

In order to obtain the desired selective effect, the yeast cultures had to be adapted to the disinfectants.

It has been described that after a prolonged period of cultivation varying with the strain and the substance, yeasts can be adapted to chemical agents [5]. Whether during adaptation a gradual change occurs in the properties of the cells or resistant cells are selected, is an open problem. Microorganisms generally tolerate well one exposure to irradiation, chemical agents or high temperature. However, they are usually destroyed on multiple transfers in media containing given concentrations of disinfectants.

Materials and methods

Saccharomyces cerevisiae strains V 30 and D 18 maintained in our culture collection are used for the production of alcohol from molasses by various plants in this country. The strains were adapted to formalin, active chlorine, cetylpyridinium and sodium penta-chlor phenolate.

The culture medium was inoculated with the yeast strain and with bacteria isolated from industrial mash. By live counts performed on milk medium [6], the concentration of disinfectants inhibiting bacterial growth was determined. The influence of various concentrations of disinfectants on the fermentation process was estimated in fermentation tests. The disinfectant concentration completely inhibiting the growth of the yeast was determined turbidimetrically. The effect of disinfectants is a function of the composition of the medium (concentration, pH, etc.); of the used substances this consideration has been

Table I

Adaptation of yeast to gradually increasing concentrations of disinfectants

Disinfectant	Steps of adaptation	Dilution	No. of transfers
Formalin	1	1 : 4300	10
	2	1 : 2800	10
	3	1 : 2500	20
	4	1 : 2200	30
	5	1 : 2000	150
	6	1 : 1320	130
Active chlorine	1	1 : 10000	10
	2	1 : 5000	10
	3	1 : 2500	20
	4	1 : 1700	30
	5	1 : 1000	280
Cetylpyridinium	1	1 : 20000	10
	2	1 : 13500	10
	3	1 : 10000	20
	4	1 : 8000	30
	5	1 : 6700	30
Sodium penta-chlor phenolate	1	1 : 100000	50
	2	1 : 40000	50
	3	1 : 2000	120

especially true for chlorine, the activity of which was considerably lowered immediately after addition to the medium. The amount of chlorine added was chosen with regard to the composition of the medium and the loss of activity during incubation. Immediately after addition to the medium used in the experiments, the active chlorine content decreased to 20 per cent. One hour later only 10 per cent of the added chlorine was revealed. In the Tables the actual amounts of chlorine added are presented. The real chlorine content of the medium was only $\frac{1}{3}$ of this value at addition and progressively less as incubation proceeded.

As an adaptation medium a 15 per cent filtered molasses solution supplemented with superphosphate and ammonium sulphate was used. The reaction was adjusted to pH 5 with sulphuric acid. The disinfectants were pipetted into the tubes and mixed in the medium immediately before inoculation. Transfers were performed at intervals of 3–4 days. The steps of adaptation are presented in Table I.

The tubes were continuously observed and, according to the intensity of fermentation, the amount of deposit and microscopic findings, the concentration of the disinfectant was increased. The two yeast strains behaved similarly during adaptation and so they were treated identically. Sometimes, when the cultures had become contaminated with bacteria, the pure yeast culture was regained by streak cultures on plated medium. When transfers were performed to higher concentrations of the disinfectant, tubes containing the lower concentration were also inoculated until it was certain that the strains were able to grow at the increased concentration.

The results were estimated by measuring the intensity of fermentation 6, 12, 24, 36 and 48 months after the adaptation experiments had been started. Measurements were carried out in Erlenmeyer flasks containing 500 ml of the indicated medium. The medium contained lower amounts of disinfectants than the final adapted concentrations. Carbon dioxide production was estimated from the loss of weight of the flasks. A 50 ml culture of the yeast strain served as inoculum for the 500 ml flasks. The flasks were incubated at 30° C until constant weight was reached. Cultures serially transferred in disinfectant-free media parallel with the adapted strains were used as controls. Each disinfectant was examined in 2 to 4 parallel experiments. In some of the series the cell count (dry weight), residual sugar (BERTRAND's method [8]), and distillable alcohol content were determined.

Results

The results are shown in Figs. 1 to 8. Fig. 1 and 2 show the fermentation rates measured after 6 and 48 months' adaptation to formalin, respectively. The yeast was adapted in the former experiment to 0.0398 per cent, in the

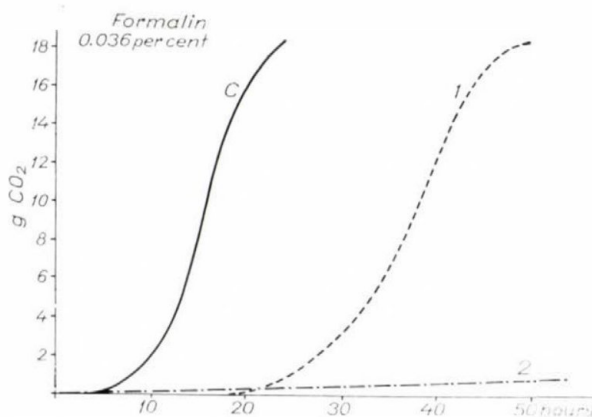


Fig. 1. Fermentation rate of yeasts after 6 months' adaptation to 0.0393 per cent formalin in the presence of 0.036 per cent formalin

latter to 0.0750 per cent, formalin. The respective concentrations used for the measurements were 0.036 and 0.050 per cent.

Figs. 3 and 4 show the fermentation by chlorine resistant yeasts after 6 and 48 months' adaptation. At the time of measurement, the yeast was

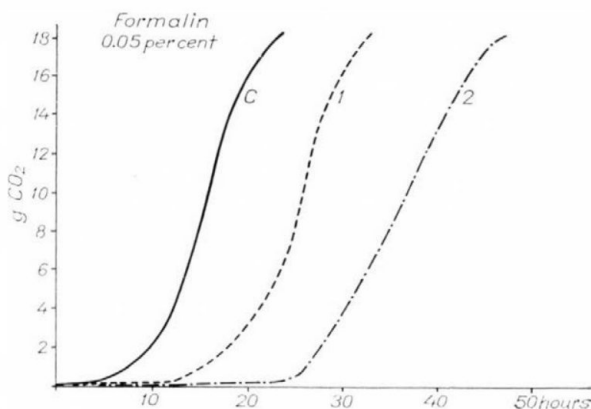


Fig. 2. Fermentation rate of yeasts after 48 months' adaptation to 0.075 per cent formalin in the presence of 0.05 per cent formalin

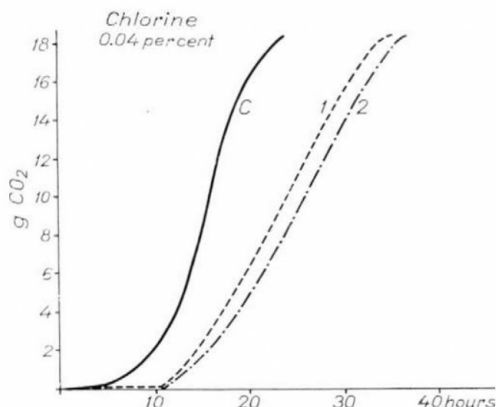


Fig. 3. Fermentation rate of yeasts after 6 months' adaptation to 0.04 per cent active chlorine in the presence of 0.04 per cent active chlorine

adapted to 0.04 and 0.10 per cent chlorine. The respective concentrations applied for the measurements were 0.04 and 0.075 per cent.

In Figs. 5 and 6 the adaptation to cetylpyridinium after 6 and 24 months is presented. Adapted concentrations were 0.0125 and 0.0150 per cent. The concentration used for the measurements was 0.010 per cent for both cultures.

The result of sodium penta-chlor phenolate adaptation after 6 and 36 months is shown in Fig. 7 and 8. The two strains are presented together, as, within the limits of error, they yielded the same result. Adapted concentrations were 0.001 and 0.005 per cent. Measurements were carried out at 0.001 and 0.002 per cent. The fermentative capacity of the yeast remained unaltered,

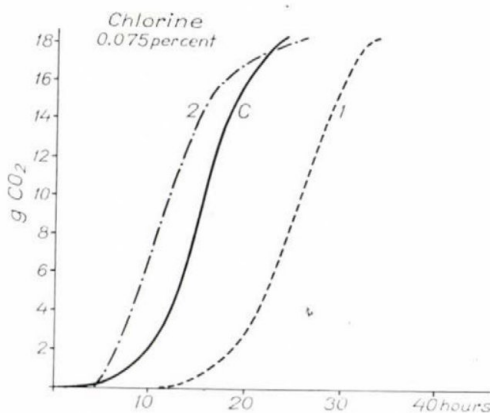


Fig. 4. Fermentation rate of yeasts after 48 months' adaptation to 0.10 per cent active chlorine in the presence of 0.075 per cent active chlorine

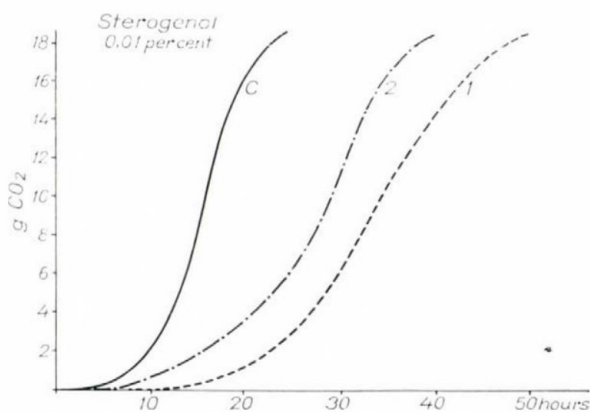


Fig. 5. Fermentation rate of yeasts after 6 months' adaptation to 0.0125 per cent cetylpyridinium in the presence of 0.01 per cent cetylpyridinium

that is, was the same as that observed with the control, at an 0.001 per cent sodium penta-chlor phenolate concentration. At 0.002 per cent concentration fermentation was delayed. As a result of the adaptation, the time of fermentation decreased from 40 to 25 or 35 hours in the presence of 0.002 per cent sodium penta-chlor phenolate.

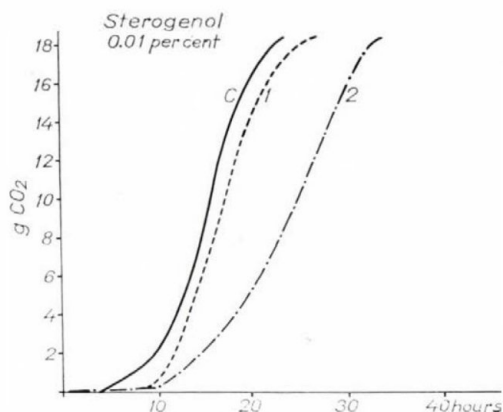


Fig. 6. Fermentation rate of yeasts after 24 months' adaptation to 0.015 per cent cetylpyridinium in the presence of 0.01 per cent cetylpyridinium

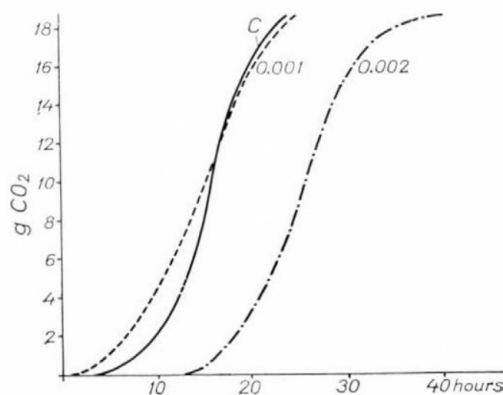


Fig. 7. Fermentation rate of yeasts after 6 months' adaptation to 0.001 per cent sodium penta-chlor phenolate in the presence of 0.001 and 0.002 per cent sodium penta-chlor phenolate

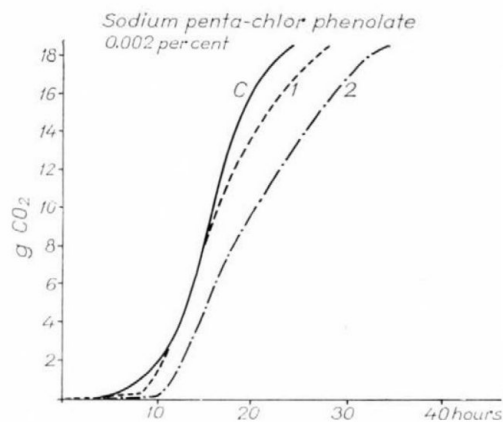


Fig. 8. Fermentation rate of yeasts after 36 months' adaptation to 0.005 per cent sodium penta-chlor phenolate in the presence of 0.002 per cent sodium penta-chlor phenolate

The control curve (C) shows the fermentation rate of unadapted yeasts in a medium free from disinfectants. As, within the limits of error, the two strains behaved similarly, the control curve was plotted from the average of all experiments. Curve 1 concerns strain V 30, curve 2 strain D 18. As in mash without disinfectants, adapted strains behaved almost identically with the control, their fermentation results were omitted. The behaviour of the control strains in the disinfectant-containing mash was examined in every experiment. It was shown that, at the concentrations used, unadapted strains were either unable to exert any fermentative activity, or they started to ferment after a considerable delay, generally when fermentation had already been completed, in parallel cultures inoculated with the adapted yeasts.

The results of fermentation experiments with adapted strains as to yeast residual sugar and alcohol content are shown in Table II. Adaptation took 48 months in case of chlorine and formalin and 36 months in the case of sodium penta-chlor phenolate.

Table II
Fermentation products obtained with adapted yeasts

Disinfectant	Concentration g/100 ml	30% dry weight yeast content g/100 ml		Residual sugar as glucose g/100 ml		Alcohol ml/500 ml		Time of complete fermentation, hours	
		V30	D18	V30	D18	V30	D18	V30	D18
Control	—	2.90	2.85	0.13	0.13	24.00	24.00	25	24
Formalin	0.05	2.70	2.50	0.13	0.15	24.00	23.65	31	48
Active chlorine	0.075	2.95	2.40	0.15	0.18	23.77	24.00	40	24
Sodium penta-chlor phenolate	0.002	2.06	2.15	0.11	0.13	23.90	23.90	29	32

Discussion

From the results it is clear that the adaptation experiments have been successful. The adapted yeasts are able to ferment at disinfectant concentrations completely or strongly inhibiting the metabolism of unadapted cultures. Adaptation is a slow process. At the beginning, after 6 months, the yeasts are considerably less resistant than after 12, 36 or 48 months. In media free from disinfectants the behaviour of adapted yeasts is identical with that of unadapted strains. In the presence of disinfectants due to a prolonged lag phase, the start of fermentation is delayed. However, the fermentation rate in the logarithmic phase is identical with that of the control. The time needed for complete fermentation by adapted strains is generally longer in the presence of disinfectants than that required by the control culture, in the absence of

those substances. The yeast, alcohol and residual sugar content of the medium is practically the same in both cultures.

With adaptation to formalin a 3-fold, to chlorine a 10-fold, to cetylpyridinium a 3-fold and to sodium penta-chlor phenolate a 5-fold increase in resistance was obtained. The fermentation rate experiments were performed at lower concentrations, since, although these amounts were tolerated, otherwise the lag phase and thus the whole fermentation time would have been unadequately prolonged.

Our formalin adapted strain was examined by ROSA *et al.* in the Warburg apparatus at 0.01—0.05 per cent formalin concentrations [9]. They showed that although originally 0.01 per cent was inhibitory, after adaptation the yeast was capable of withstanding a concentration of 0.05 per cent. The Warburg experiments cannot, of course, be compared with our fermentation rate experiments. In the former the conditions are much less favourable, as fermentation is measured at the beginning of cultivation in the delayed phase.

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STUDIES ON THE AETIOLOGY OF EPIDEMIC KERATOCONJUNCTIVITIS OBSERVED IN BUDAPEST DURING WINTER, 1961—1962

By

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Summary. Attempts were made to reveal the aetiological factor of epidemic keratoconjunctivitis observed in Budapest in winter, 1961—1962, by isolation of the pathogenic agent and by haemagglutination inhibition, complement-fixation, and neutralization tests.

Nine strains were isolated from conjunctival washings obtained from 58 patients, in HeLa or Detroit-6 cell cultures. The complement-fixation tests have shown that the strains belonged to the adenovirus family. According to the neutralization and haemagglutination-inhibition tests seven strains belonged to type 8, the remaining two to type 6.

Paired sera were available from 38 of the 58 patients tested for virus. A rise in the complement-fixing and neutralizing antibody titres to adenovirus type 8 was shown by 30 patients (78 per cent); in 50 per cent of the paired sera the rise was, at least, 4 fold. The convalescent titres ranged from 1:4 to 1:32. The haemagglutination-inhibition test showed an increase in titre in 36 cases (94 per cent), a 4 fold or greater rise in 34 cases (89 per cent). The convalescent titres ranged from 1:8 to 1:1024.

Serum pair was available from one of the two patients yielding adenovirus type 6; this patient also produced haemagglutination-inhibiting antibodies to adenovirus type 8.

It has been concluded that the epidemic was caused by adenovirus type 8.

The haemagglutination-inhibition test is recommended as the method of choice for the laboratory diagnosis of epidemic keratoconjunctivitis.

The discovery of haemagglutination (HA) by adenoviruses [1] provided a new method which has simplified the typing of adenovirus isolates [2]. The HA test itself can serve as a basis for determining the group which includes the serological type of an isolate. In more precise typing the HA-inhibition (HI) test can substitute the neutralization test [2, 3]. ROSEN [4] demonstrated for adenovirus type 1—7 that in the course of infection homotypic HI antibodies as specific as the neutralizing antibodies appear in the blood serum.

In winter, 1961—1962, epidemic keratoconjunctivitis (EKC) was observed in Budapest. We isolated several viral agents and typed them by the HI test. Paired sera obtained from the same patients were examined by the same test. For comparison the neutralization and the complement-fixation (CF) tests were also carried out. These studies are discussed in the present report.

Materials and methods

Tissue cultures. Detroit-6 and HeLa cell cultures were used for virus isolation and, partly, for the neutralization tests. The cells were transferred by trypsinization as usual. The growth medium for the HeLa cells contained 40 per cent calf serum, 30 per cent Hanks' balanced solution, and 30 per cent Parker's 199; that for the Detroit-6 cells consisted of 10 per cent rabbit serum and 90 per cent Hanks' balanced solution to which 0.4 per cent lact-

albumin hydrolysate had been added. The maintenance solution for the Detroit-6 cultures was 5 per cent rabbit serum and 95 per cent Hanks' balanced solution containing 0.4 per cent lactalbumin hydrolysate; that for HeLa cultures, Parker's 199. Antibiotics were added as usual. In part of the neutralization tests human primary amnion cell cultures were applied. These were prepared as published elsewhere [5].

The HA technique and the treatment of the erythrocytes [6] as well as the preparation of immune sera [7] were also described.

HI tests. TAKÁTSY's Microtiter [8] was applied. Each test was preceded by HA titration; a twofold dilution series of virus was prepared in saline, and the same volume of a 1 per cent rat erythrocyte suspension was added. The test was read after 30 minutes incubation at room temperature. The HI test mixture contained the given dilution of serum and 4 HA units of virus in a volume of 0.025 ml. The mixture was allowed to stand at room temperature for 30 minutes, then one drop (0.025 ml) of a 1 per cent rat erythrocyte suspension was added. After another 30 minutes the test was read. As final titre the highest dilution of serum showing complete inhibition was registered. Serum samples agglutinating the test erythrocytes in dilutions higher than 1:8 were absorbed with the erythrocytes before used in the test.

The neutralization test and the complement-fixation test were carried out as described earlier [9, 10].

Results

Virus isolation was attempted from 58 conjunctival washings collected from three affected districts of Budapest. Each specimen was inoculated into three Detroit-6 cell cultures, and these were observed, in general, for two weeks. Then the medium was subcultured regardless of whether cell degeneration had or had not been observed. After three passages in Detroit cells HeLa cultures were inoculated. These were slightly more sensitive to adenoviruses. The further passages were carried out in HeLa cells. In general, five or six blind passages were made. Of the lines thus obtained only those showing any kind of cytopathic effect (CPE) were passaged further, until the CPE had been fixed, *i.e.* until the virus strain had been established. Table I shows

Table I

The development of CPE by a strain (M. I.) of adenovirus type 8

Passage No.	Cell culture	Incubation time day	CPE*	
			degree	character
1	Detroit-6	15	±	uncertain
2	„	14	++	uncertain
3	„	11	+	uncertain
4	HeLa	13	+++	uncertain
5	„	15	++++	typical
6	„	14	++	uncertain
7	„	14	++++	typical

* = based on three cell cultures

+ = degenerative foci

++ = degeneration in 50 per cent of cells

+++ = degeneration in 75 per cent of cells

++++ = complete degeneration

the history of one of the adenovirus type 8 isolates. The adaptation of these strains to the cells proceeded slower than the adaptation of adenoviruses in general. In the case of the M. I. strain the CPE had not been complete by the end of passage 4 (*i.e.* on the 53rd day of cultivation); at the same time the character of the CPE was indefinite. The degeneration had become complete as late as in passage 5 (68th day of cultivation). In passage 6 CPE became weaker again. In the subsequent passages CPE was complete and typical. The other type 8 strains behaved similarly.

All the the nine strains fixed complement in the presence of the common adenovirus antigen and agglutinated rat erythrocytes. HA appeared as soon as in the 3rd or 4th passages, *i.e.* much earlier than the CPE had stabilized.

Further typing was made by the HI test (Table II). Of the nine isolates seven proved to be of type 8 strain, the remaining two belonged to type 6. These results were verified by the neutralization test.

Table II
Typing by the HI test

Type serum	Strain										Type strain	
	F. J.	M. I.	B. Gy.	F. G.	Sz. L.	T. J.	P. D.	J. F.	Mé. I.		adeno 8	adeno 6
adeno 3	—	—	—	—	—	—	—	—	—	—	—	—
„ 4	—	—	—	—	—	—	—	—	—	—	—	—
„ 6	—	—	—	—	—	—	—	+	+	—	—	+
„ 7	—	—	—	—	—	—	—	—	—	—	—	—
„ 8	+	+	+	+	+	+	+	—	—	+	—	—
„ 10	—	—	—	—	—	—	—	—	—	—	—	—
convalescent (type 8)	+	+	+	+	+	+	+	—	—	+	—	—
convalescent (type 6)	—	—	—	—	—	—	—	+	+	—	—	+

⊕ = inhibition

— = no inhibition

Table III presents the antibody responses to adenovirus type 8 by six out of the nine patients who yielded virus. In the acute-phase serum specimens neither HI nor neutralizing antibodies were detectable; CF antibodies supposedly having resulted from earlier infections with other types of the adenovirus family were demonstrable in the acute-phase specimens of two patients. All the six patients produced antibodies demonstrable by each of the three tests applied. In the convalescent specimens the HI titres highly exceeded the neutralization titres and the CF titres. It should be noted that Patient No. 3

Table III*Antibody responses to adenovirus type 8 of the patients who yielded virus*

	Paired sera	HI	CF	Neutraliza- tion
1. M. I.	acute convalescent	— 16	— 4	— 8
2. F. G.	acute convalescent	— 64	— 4	— 4
3. J. F.*	acute convalescent	— 32	— 8	— 16
4. B. Gy.	acute convalescent	— 1024	4 16	— 8
5. Sz. L.	acute convalescent	— 64	— 4	— 32
6. F. J.	acute convalescent	— 128	4 16	— 32

— = negative in 1 : 4

* = adenovirus type 6 was isolated

(J. F.), from whom adenovirus type 6 had been isolated, also produced type 8 antibodies.

We extended the serological study to the paired sera of 29 patients who did not yield virus. The acute-phase specimens were taken, in general, on the 2nd to 4th days of illness, the convalescent specimens three weeks thereafter. The results obtained from testing all the 38 serum pairs are summarized in Table IV.

Table IV*HI, neutralization and CF antibodies in the paired sera of 38 patients*

Titre	HI		Neutralizing		CF	
	acute	convalescent	acute	convalescent	acute	convalescent
negative*	30	2	36	8	25	8
1 : 4	—	—	2	3	13	6
1 : 8	3	4	—	9	—	11
1 : 16	2	3	—	11	—	9
1 : 32	2	8	—	7	—	1
1 : 64	—	8	—	—	—	—
1 : 128	1	4	—	—	—	—
1 : 256	—	2	—	—	—	—
1 : 512	—	3	—	—	—	—
1 : 1024	—	4	—	—	—	—

* = negative in 1 : 4

These data and the further analysis of the results have shown that 34 of the 38 patients (89 per cent) elicited an at least 4 fold rise in the HI titre. In two serum pairs no HI antibody was found in another two the increase was twofold. The HI test carried out with another type 8 strain showed fourfold increase for the last two serum pairs too. If the rise of these two is also regarded as significant, the HI antibody response of 94 per cent of the patients tested can be accepted as positive for adenovirus type 8. The positive convalescent titres ranged from 1 : 8 (four specimens) to 1 : 1024 (in 13 samples 1 : 128 or higher). On the other hand, of the acute-phase specimens only 8 contained a detectable amount of HI antibodies; the basal dilution was 1 : 4.

The CF and the neutralization titres increased in 30 serum pairs (78 per cent); the increase was fourfold or higher in about 50 per cent of the paired sera. The convalescent titres ranged from 1 : 4 to 1 : 32 (mostly from 1 : 4 to 1 : 16).

Discussion

The above results seem to allow the conclusion that the epidemic under study was caused, first of all, by adenovirus type 8. The low isolation rate (7/58) for this virus is in accordance with the following findings of other investigators. (i) The isolation of adenovirus type 8 required a relatively long time, *viz.* 21—29 [11, 12] or 31—62 [13] days; (ii) only very few infectious particles were obtainable from even the most acute cases of EKC [13]; (iii) the tissue cultures infected by adenovirus type 8 (including our strains) and those isolated from EKC patients in spring, 1962, in Szeged [14] produced less than 300 TCID₅₀ of virus per ml. The low titres may be explained by the observation of JAWETZ *et al.* [13, 15]. These authors found that in the cell cultures usually applied to cultivate adenovirus type 8 only a small part of the virus yield demonstrable by the CF test was infectious.

The fact that most of the patients yielding no virus, and one from whom type 6 virus was isolated, showed a marked antibody response to adenovirus type 8 confirms the leading role of this virus in the epidemic under study. The same conclusion has been drawn by others for numerous epidemics during the most recent years.

The aetiological importance of adenovirus type 8 in the Budapest epidemic is supported by IMRE, KORCHMÁROS and GECK [23], who carried out immunofluorescence studies, and by BÉLÁDI *et al.* [14], who isolated this virus from cases of EKC observed in Szeged in May, 1962. On the other hand, it should be mentioned that at the same time a herpesvirus strain was isolated from a case of EKC in Debrecen [24].

The pathogenicity of adenovirus type 8 was further confirmed by three accidental laboratory infections observed in this laboratory. These cases

produced typical keratoconjunctivitis with corneal infiltration, which was one of the most dominant symptoms during the epidemic [25].

Type-specific HI antibodies were generally produced to adenovirus type 8 during the illness. These antibodies were as specific as the neutralizing antibodies. On the other hand, the HI test appeared to be superior to the neutralization and the CF tests from several regards, *viz.* (i) 3—4 weeks after onset the HI test was positive in a higher percentage; (ii) the rise in titre was much more significant; (iii) the HI test is considerably simpler, cheaper and less time-consuming than the neutralization test. Consequently, the HI test can be accepted as an appropriate test for studying the aetiology of EKC (and probably, other diseases of adenovirus origin).

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SUPPLEMENTED PHAGE-TYPING OF SALMONELLA TYPHI MURIUM AND ITS USE IN EPIDEMIOLOGY

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Summary. A total of 1895 *S. typhi murium* strains has been typed by FELIX and CALLOW's phages. It has been shown that, because of the frequent occurrence of certain phage types, untypable and degraded cultures, the typing method of FELIX and CALLOW is not sufficient for epidemiological purposes.

As a supplement, KRISTENSEN's biochemical typing simplified by KALLINGS was applied. In addition, the properties of temperate phages of the cultures were determined.

As determined by FELIX and CALLOW's method, the most frequent phage types according to foci were 2b, 4, 5, 1a and 1a var. 1. Types 1, 1b, 2, 2a, 2c and 3 and other, possibly distinct types that could not be fitted into the scheme, were also encountered. Of the examined strains 76.6 per cent were lysogenic.

Within the phage types a further subdivision was made on the basis of the properties (lytic activity, heat resistance) of temperate phages. A comparison of epidemiological data with the direct typing method supplemented with the identification of temperate phages and biochemical behaviour of the strains has shown that the combined method may be of value for epidemiological purposes.

The frequent occurrence of epidemic and sporadic *S. typhi murium* infections in Hungary has justified the introduction of routine phage typing of that organism.

A method for the phage typing of *S. typhi murium* has been elaborated by FELIX and CALLOW [1, 2]. Their method was based on a phage isolated from the faeces of a patient suffering from food poisoning. The phage lysed the *S. typhi murium* strain excreted by the same person. The 12 typing phages of the FELIX—CALLOW scheme were obtained partly from lysogenic strains, partly by adaptation. A detailed scheme was published by FELIX in 1956 [3], then by CALLOW in 1959 [4]. In CALLOW's paper 34 types were distinguished by use of 31 phages and the elaboration of an improved scheme was foreshadowed. For practical purposes the differentiation of more and more types was needed. Without the standardization of the method, by new typing phages ANDERSON and WILSON distinguished 90 types [5].

A different scheme was established by LILLENGEN [6], who divided the *S. typhi murium* strains occurring in Sweden into 24 types by 12 phages.

BOYD [7, 8] classified *S. typhi murium* into types on the basis of "symbiotic" (temperate) phages. He has shown that lysogeny is a constant property of the strains and it may be used for epidemiological purposes. The "symbiotic" phages of *S. typhi murium* corresponded to BURNET's types A

Table I

Reactions of type strains of Salmonella typhi murium with routine test dilutions of the typing phages

Type strains	Typing phages												
	1	1a	1a var. 1	1b	2	2a	2b	2c	2d	3	3a	4	5
1	OL	OL	OL	<CL	CL	CL	>CL	CL	OL	OL	OL	±	OL
1a	—	OL	OL	<CL	CL	<CL	OL	<CL	OL	OL	OL	—	OL
1a var. 1	—	OL	<CL	<CL	—	±	OL	—	OL	OL	<CL	—	++
1b	—	—	<CL	<CL	CL	CL	OL	<CL	OL	<CL	<CL	—	—
2	—	—	—	—	CL	CL	—	CL	—	—	—	—	—
2a	—	—	—	—	—	CL	>CL	CL	>CL	—	—	—	—
2b	—	—	—	—	—	—	OL	—	OL	—	—	—	—
2c	—	—	—	—	<CL	<CL	<CL	<CL	<CL	—	—	—	—
2d	—	—	—	—	±	±	±	±	>CL	—	—	—	—
3	—	—	±	+++	—	—	—	—	—	OL	++	—	—
3a	—	—	±	—	—	—	+++	—	SCL	+	>CL	—	—
4	—	—	—	—	—	±	—	±	±	—	—	>CL	—
5	—	—	—	++	—	—	—	—	—	++	—	—	OL

and B [9], which were found in other salmonellae, too. The types A and B described by BOYD were distinguished by differences in the size and appearance of plaques, heat resistance and serological properties. The symbiotic phages might be used as markers for the identification of the strain. Later BOYD *et al.* [10] reported on the successful epidemiological application of the method.

Of the typing methods, that of FELIX and CALLOW's is the one most extensively used in practice. However, both in general use and applicability, it is inferior to the phage typing methods worked out for *S. typhi* and *S. paratyphi B*. The international standardization of the method is hindered by the large number of types and their different distribution in various countries.

In this laboratory the typing of *S. typhi murium* was started in 1960 by means of FELIX and CALLOW's 13 phages. It was soon realized that because of the frequent occurrence of certain types and untypable or degraded strains, FELIX and CALLOW's method is not sufficient for epidemiological investigations. Therefore biochemical typing and examination of temperate phages were also introduced.

Materials and methods

The phage type, biochemical type and lysogeny of 1895 strains isolated mainly from patients, less frequently from contact carriers, food samples and domestic animals, were examined.

Phage typing was carried out according to the FELIX—CALLOW scheme. Typing phages were obtained from the International Committee for Enteric Phage Typing. The method was the same as that used for the typing of *S. typhi* (Table I).

For biochemical classification the scheme of KRISTENSEN *et al.* [12] and HARHOFF [13] simplified by KALLINGS and LAURELL [11] was used. KRISTENSEN *et al.* and HARHOFF distinguished 20 kinds of biochemical types by taking into consideration the reactions in rhamnose,

Table II
Biochemical types of S. typhi murium

Simplified biochemical types	Rhamnose	Inositol	Stern broth	Bitter xylose	Biochemical types of Kristensen
1	+	+	+	+	1 (6)
2	+	+	+	—	2 (3, 4, 5)
3	+	+	—	+	7
4	+	+	—	—	8
5	+	—	+	+	9
6	+	—	+	—	10 (11, 12)
7	+	—	—	—	(13)
8	—	+	+	—	(14, 19, 20)
9	—	—	+	—	(15, 16) 17
10	—	—	—	—	18

Bracketed figures indicate rare biochemical types

inositol, Stern glycerol, d-, l-, i-tartrate, sodium citrate, Simmons glucose and Bitter xylose. KALLINGS and LAURELL distinguished 10 biochemical types on the basis of behaviour in Stern glycerol, Bitter xylose, rhamnose and inositol. The reactions given by the 10 biochemical types are shown in Table II.

Lysogeny. For liberating temperate phages the somewhat modified method of BOYD was used [7, 8]. The culture to be examined and the inducing strain (BOYD's non-lysogenic *S. typhi murium* strain 1404) were inoculated into broth. After incubation for 18 hours at 37° C the culture was centrifuged and the supernatant was divided into 4 aliquots, which were then treated as follows. (1) Left untreated; (2) Heated at 58° C for 30 minutes; (3) Heated at 70° C for 30 minutes; (4) Treated with 0.1 per cent toluol at 37° C for 30 minutes, then freed from toluol by evaporation under vacuum.

The purpose of the different treatments was the destruction of bacteria and differentiation between phages of various heat sensitivity. Separation of heat sensitive phages was performed with toluol instead of the troublesome method of filtering. The four kinds of fraction were examined with indicator strains, including BOYD's non-lysogenic *S. typhi murium* cultures 1404 and 1411 and 7 further arbitrarily chosen salmonellae: *S. typhi murium* strains M22, M33, M55 (isolated from patients), non-lysogenic *S. paratyphi B* strains 3aB62 and Scholtens' 4096 and laboratory strains of *S. paratyphi A* and *S. abortus equi*. Agar plates were flooded with the 2 hour cultures of the indicator strains and aliquots of the 4 different fractions were dropped onto the media. Readings were made after an incubation at 37° C for 8 hours.

Table III
Percentage distribution of *S. typhi murium* types
according to number of strains and foci

Phage type	1960		1961		1962		1960-1962	
	Strain	Focus	Strain	Focus	Strain	Focus	Strain	Focus
1	1.8	2.0	3.9	6.8	5.8	2.2	4.6	4.1
1a	34.3	18.0	14.8	15.8	8.6	10.4	13.0	13.1
1a var. 1	14.4	22.0	22.2	9.8	21.1	10.2	21.2	10.8
1b	7.2	12.0	0.2	0.3	4.5	2.9	2.6	2.4
2	—	—	0.9	1.5	0.1	0.2	0.5	0.8
2 var. 1	1.8	—	—	—	—	—	0.1	—
2 var. ?	1.8	—	9.9	0.9	—	—	4.8	0.4
2a	—	—	0.2	0.6	1.1	1.9	0.6	1.2
2b	1.8	4.0	10.9	16.3	19.3	24.3	14.2	19.5
2b var. ?	7.2	4.0	0.1	—	—	—	0.5	0.2
2b degr.	—	—	2.6	3.3	0.2	0.5	1.3	1.6
2c	0.9	2.0	2.0	3.0	2.1	3.9	2.0	3.4
3	—	—	0.1	0.3	—	—	0.1	0.1
3a	—	—	1.1	1.2	0.1	0.2	0.6	0.6
4	2.7	4.0	10.9	19.3	13.1	18.4	11.5	17.9
5	2.7	6.0	13.2	12.5	19.8	18.6	15.7	15.4
degraded	—	—	2.1	4.5	1.5	2.7	1.7	3.3
untypable	23.4	26.0	4.9	3.9	2.7	3.6	5.0	5.2
No. of strains	111		901		883		1895	
No. of persons	107		672		669		1439	
No. of foci	50		336		413		799	

Results

Of the 13 types described by FELIX and CALLOW 12 were encountered. The distribution of types is presented according to the number of strains and foci (Table III). According to foci the most frequently occurring types were 2b, 4, 5, 1a and 1a var. 1.

Untypable cultures occurred in 5 per cent of the total number of strains or in 5.2 per cent of the foci.

Of the strains 2 var. ?, 2b var. ?, 2b degraded, and degraded, cultures could not be included in the scheme (altogether 8.3 per cent of the total number and 4 per cent of the foci). These strains deviated from the typical lytic patterns of the scheme. It might be assumed that they corresponded to distinct types, as the observed deviations were constant and epidemiological findings also indicated their common origin.

More frequently occurring phage patterns that could not be fitted into the scheme are shown in Table IV. Strains lysed by phages 2, 2a, 2c and 4 are not included in the Felix—Callow scheme; their occurrence, however, was noted by FELIX [2] and RISCHÉ *et al.* [14], who designated them as 2 var. 1. Similar cultures were also met with in the course of the present work.

Table IV

*More frequently occurring phage patterns not included in the Felix—Callow scheme**

	Typing phages												
	1	1a	1a var. 1	1b	2	2a	2b	2c	2d	3	3a	4	5
1	SCL	CL	CL	CL	—	—	SCL	—	SCL	SCL	SCL	—	CL
2	—	+++	+++	—	+	±	SCL	+	CL	—	—	—	++
3	—	—	—	—	++	±	—	—	—	—	—	++	—
4	—	—	—	—	SCL	±	CL	—	CL	—	—	±	—
5	—	—	—	—	CL	CL	—	CL	—	—	—	+	—
6	—	—	—	—	+++	—	—	—	—	—	—	±	—
7	—	—	—	—	+	—	+++	—	+++	—	—	—	—
8	—	—	—	—	+	—	—	—	—	—	—	++	—
9	±	+	—	—	—	—	CL	—	CL	—	—	+	++
10	±	+	—	—	—	±	CL	+	CL	—	—	—	—
11	—	±	—	—	—	—	CL	—	CL	—	—	±	—

Degrees of lysis:

CL = Confluent lysis

SCL = Semiconfluent lysis

± to +++ = Increasing numbers of discrete plaques

* Strains with phage patterns shown in this Table were included in Table III as types var. ?, 2b var. ?, 2b degraded, and degraded.

The result of classification according to biochemical reactions is presented in Table V.

Phage type 1 can be divided into 3, type 1a into 5, the frequently occurring type 2b into 4 and untypable cultures into 4 different biochemical types.

Table V
Frequency of biochemically subdivided S. typhi murium phage types

Phage type	Biochemical type									Total
	1	2	7	8	9	10	13	17	Nc	
1	15				4	1				20
1a	40	13			46	26	2			127
1a var. 1	8	1			100	31				140
1b	8									8
2	7	1	1	1						10
2a	1							2		3
2b	71	2			3				1	77
2b degr.	5				1					6
2b var. ?	9									9
2c	6	2			6	1				15
3					1					1
4	14	4	22	8		1				49
5					67				1	68
degraded	3	3	1		2				1	10
untypable	53		2		6	3				64
Total	240	26	26	9	236	63	2	2	3	607

Nc = Not conforming to any of the described biochemical types

Of the mentioned cultures that failed to correspond to typical lytic patterns, "2b var. ?" strains, were uniform not only in phage sensitivity but also in biochemical behaviour.

In some of the cases the biochemical reactions gave unreliable results.

Of the examined *S. typhi murium* strains 76.6 per cent were lysogenic. The distribution of lysogenic strains according to phage types is presented in Table VI.

Types 1b, 2a, 2b, 2c and 5 were lysogenic without exception. Types 2, 1a and 4 were lysogenic in 20, 28 and 55 per cent, respectively. The frequent lysogeny of degraded and untypable strains was useful for the differentiation between these commonly occurring cultures.

The properties of temperate phages were characterized by their lytic activity and heat sensitivity. With toluol treatment heat sensitive phages were

Table VI
Distribution of phage types according to lysogeny

Phage type	Total no. of strains	No. of lysogenic strains	No. of groups distinguishable by temperate phages
1	24	17	12
1a	157	44	28
1a var. 1	168	155	35
1b	9	9	3
2	10	2	2
2a	6	6	4
2b	104	104	52
2b degr.	8	8	5
2b var.	9	9	1
2c	18	18	12
3	1	—	—
3a	2	—	—
4	81	45	34
5	118	118	33
degraded	14	14	9
untypable	75	68	36
Total	804	617	

obtained. Other phages were divided into two groups by heating at 58° and 70° C.

Table VI indicates the number of groups established on the basis of host range and heat sensitivity of temperate phages. Thus in type 1a 28, in 1a var. 1 35, in 2b 52, in 2c 12, in 4 34, in 5 33 and among untypable cultures 36 further subtypes or groups were distinguished.

In addition to differences in host range and heat sensitivity, the size of plaques also varied. The more heat resistant, large plaque-forming phage A and the less heat resistant, small plaque-forming phage B of BURNET [9], were, however, differentiated in course of large scale typing only by heat sensitivity testing.

Comparison of classification based on phage typing, biochemical reactions and lysogeny with epidemiological data. Altogether 104 type 2b strains were examined. In Fig. 1 strains with obtainable epidemiological history are classified by the properties of their temperate phages.

From 40 strains heat resistant phages surviving an exposure to 70° C were liberated. The host range of these phages was uniform. Of the strains 20 were isolated from an outbreak of ice cream poisoning. From a larger epidem-

ic possibly originating from one focus, 11 strains were obtained. Nine further strains were isolated from sporadic cases (lytic pattern B). A home outbreak yielded 7 strains with identical temperate phages (lytic pattern C). Two strains with lytic pattern F and two with lytic pattern G were isolated from food poisoning including a home outbreak. These strains behaved uniformly on both phage and biochemical typing; the further division into several groups

Designation of temperate phage type	Indicator strains								Number of strains
	<i>S. typhi</i> murium					<i>S. paratyphi</i> B 3a	<i>S. paratyphi</i> B 4096	<i>S. paratyphi</i> A	
	22	33	55	1404	1411				
A									2
B									40
C									7
D									2
E									3
F									2
G									2

Notes:

Heat sensitivity of temperate phages.



Heat sensitive



No lysis



Resists heating at 58°C for 30 minutes



Resists heating at 70°C for 30 minutes

Fig. 1. Distribution of *S. typhi* murium strains phage type 2b according to temperate phage-properties

corresponding to epidemiological findings was possible only by temperate phages.

In July, 1959, cases of enteric infections occurred among infants in a country hospital department. From the faeces of 4 infants and one mother phage type 1a strains were isolated. The infection was probably spread by the mother. At the same time a phage type 4 *S. typhi* murium strain was isolated from the faeces of a male patient, who, accordingly, was not connected with the hospital outbreak. Neither type 1a nor type 4 strains were lysogenic. All type 1a strains but one belonged to biochemical type 9, the exceptional type 1a strain was a member of biochemical type 13. As revealed later, this

strain was in reality not isolated from any of the newborn infants, but was harboured by a 2 year old child admitted to an other department. The phage type 4 strain belonged to biochemical type 1.

A *S. typhi murium* outbreak of unknown source occurred in a day nursery. Neither of the isolated 6 strains corresponded to any of the phage types included in the scheme. The phages isolated from the strains were uniform in heat resistance and host range. The strains themselves belonged to biochemical type 1.

Following a grave outbreak of ice cream poisoning, *S. typhi murium* was isolated from the faeces of patients, from the incriminated ice cream, the floor of the confectionery, the ice cream stand, and also from the faeces of the confectioner. The isolated 20 strains all belonged to phage type 2b; their temperate phages showed the same host range and heat resistance (unsensitive to 70° C). Biochemically, they belonged to type 1. One week later in the same district new *S. typhi murium* infections occurred. The result of phage typing excluded the possibility of contact transmission, as the new strains, unlike those isolated from the ice cream-borne outbreak, belonged to phage type 2c. The two kinds of strains differed also as to temperate phages and biochemical reactions.

In another ice cream-borne outbreak, of the examined 11 *S. typhi murium* strains 7 belonged to type 2b, while 4 could not be included in the scheme. The common origin of the strains was confirmed by showing their identical biochemical behaviour and temperate phages.

From a home outbreak 13 *S. typhi murium* phage type 1a strains were isolated. Biochemically, these strains differed from the above mentioned non-lysogenic type 1a cultures. Moreover, they were lysogenic, yielding identical temperate phages. Prior to the outbreak, members of the family consumed jelly. The *S. typhi murium* strain isolated from the jelly was similar to the faecal strains in both biochemical behaviour and properties of its temperate phages.

Several strains isolated from outbreaks and case-to-case infections were typed by the combined method. The phage type of the strains originating from the same source, apart from some unimportant alterations (degradation), was identical and constant. Strains that could not be included in the scheme occurred frequently. The biochemical reactions were sometimes doubtful. As shown by repeated examinations, the lysogenic state of the strains was constant. In many cases the identity of degraded and biochemically doubtful strains was confirmed by the result of lysogeny test.

Thus, it may be concluded that phage typing supplemented with the examination of temperate phages and biochemical behaviour of the strains is a procedure most valuable for epidemiological purposes.

Discussion

The importance of lysogeny in typing has been proved for *S. typhi* *murium* by BOYD [7, 8], and for *S. paratyphi B* by SCHOLTENS [15]. ANDERSON and FELIX [16] showed that different phage types of *S. typhi* may carry identical phages and phage specificity is not determined exclusively by the temperate phage but also by its carrier, the host cell. Thus a simple determination of temperate phages would not be sufficient for type determination.

In a review ANDERSON [17] has stressed that for the characterization of a bacterium by the phage method, the determination of both phage sensitivity and temperate phages should be performed.

FELIX [3] compared the results obtained in typing by different series of adapted and Lilleengen's phages. He concluded that for epidemiological investigations no international standard method was needed, as all the different typing schemes gave adequate results. The existing different schemes may be regarded as starting points for the elaboration of methods most suitable for local requirements.

Our studies on the phage typing of *S. paratyphi B* [18] indicated that the direct FELIX—CALLOW typing method supplemented with the identification of temperate phages gave better results than the separate employment of any of the two methods.

Similarly, as shown by examples in this paper, the FELIX—CALLOW method combined with the determination of BOYD's "symbiotic" phages ensured much better results than direct typing alone.

There is general agreement that, to make it suitable for epidemiological examinations, FELIX and CALLOW's direct typing method should be improved. RISCHE *et al.* [19] pointed out that a further subdivision of the FELIX—CALLOW scheme was advisable. SPANO *et al.* [20] reported that out of 67 outbreaks in Italy, the causative agents of only 42 could be typed. NESTORESCO *et al.* [21], KALLINGS *et al.* [22], RISCHE and KRETZSCHMAR [23] and SEIDEL [24] supplemented phage typing with biochemical reactions. They showed that, although biochemical methods were adequate for the subdivision of certain phage types, with the most frequently occurring types no satisfactory differentiation was obtained. The present studies also show that biochemical reactions are of little help in case of the very types the subdivision of which would be most important (See Table V).

As 21.7 per cent of their *S. typhi murium* strains behaved atypically or was phage resistant, POPOVICI *et al.* [25] supplemented the FELIX—CALLOW scheme with 6 "local" phages.

KALLINGS *et al.* [22] observed alterations in strains isolated from food samples and patients. They found 4 strains of different phage sensitivity in one and the same faecal sample. By infecting types 7 and 8 of these strains

with temperate phages, they were able to change them into the other two phage types present in the same faeces. Temperate phages of strains isolated from the outbreak in question, exerted identical lytic activities on 50 *S. typhi murium* strains.

Of the 33 types of CALLOW's new typing scheme 26 were found lysogenic [4]. It has been pointed out that for the most accurate characterization of strains both phage sensitivity and lysogeny should be examined.

Among strains isolated from human infections BOYD [7] found 80 per cent of lysogenic cultures. This frequency stands very close to that obtained in the present study (76.6 per cent).

The heat sensitivity of a given temperate phage often differed according to the various indicator cultures. It was assumed that the corresponding strains carried two or more temperate phages differing in heat sensitivity. The possibility, however, of that the heat tolerance of the same temperate phage varied with the indicator strains, could not be excluded. Investigations into this problem are in progress.

Although the value of direct typing supplemented with the indirect method is generally accepted, the combined method is not widely used. This is possibly due to the fact that the identification of temperate phages is a troublesome and labour consuming method.

In this laboratory, the combined typing procedure is used in the following cases.

(a) When direct typing shows the occurrence of different phage types within one outbreak.

(b) When an outbreak is caused by a frequently occurring phage type and when it is to be considered whether infections encountered after the outbreak are contact cases or originate from a new source.

(c) In food poisoning, for the comparison of strains isolated from faecal and food samples.

(d) When the suspected source of infection is an animal, for the comparison of strains isolated from the animal and from infected persons.

Finally, it should be realized that although the combined method is more complicated than direct typing alone, the results obtained by its use are much more reliable than those of direct typing.

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ACCIDENTAL LABORATORY INFECTIONS BY ADENOVIRUS TYPE 8

By

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Summary. Three cases of accidental laboratory infection are described. The adenovirus type 8 strain that caused the infections had been isolated in Budapest during an outbreak of epidemic keratoconjunctivitis. The virus was re-isolated from two patients; the third patient was treated with colloidal silver as soon as three hours after the accident. All the three patients gave a specific antibody response detectable by each of the neutralization and the haemagglutination-inhibition tests. Neither virus could be isolated from, nor antibody was produced by, the close contacts and family members tested. Some conclusions concerning the aetiology, spread, and epidemiology of epidemic keratoconjunctivitis are discussed.

During an outbreak of epidemic keratoconjunctivitis (EKC) in Budapest in the winter, 1961—1962, numerous strains of adenovirus type 8 were isolated [1]. With one of the strains an accident affecting three laboratory workers occurred in our Institute. Subsequently typical EKC developed in the three persons. To our best knowledge this was the second accident resulting in laboratory infection with adenovirus type 8; the first had been reported by JAWETZ *et al.* [2]. The present accident had the special interest that the exact time of the infection was known and that three subjects were infected at the same time. The victims, their close coworkers and family members were examined by laboratory tests. The data thus obtained are the subject of the present report.

Materials and methods

Isolation of virus was attempted in HeLa and Detroit-6 cultures from conjunctival washings taken during the acute phase of illness. The cell cultures were maintained and the haemagglutination-inhibition (HI) and neutralization tests were carried out as described in the accompanying report [1].

Observations

The data of the three infections, except the clinical data, are summarized in Table I. The clinical course of the illnesses will be published in a separate report [3].

To the data presented in Table I it should be added that each of the three subjects had dealt with adenoviruses for two or three years and with adenö-

Table I

The development of EKC in the three subjects infected accidentally

Patients	Day of infection	Beginning of conjunctival infiltration	Beginning of corneal changes	Treatment	
				Beginning	Drug
Case I	4 Sep., 1962	8 Sep., 1962	right, 20 Sep., 1962 left, 22 Sep., 1962	10 Sep., 1962	Colloidal silver Silver nitrate Naphazoline
Case II	4 Sep., 1962	10 Sep., 1962	left, 22 Sep., 1962 right, 25 Sep., 1962	untreated	
Case III	4 Sep., 1962	10 Sep., 1962	Bilateral 22 Sep., 1962	4 Sep., 1962	Colloidal silver

virus type 8 for nine months. The accident happened when a rabbit was being immunized. The three laboratory workers were bending over the rabbit when from the broken syringe undiluted virus suspension was sprayed into the eyes of each of them.

After the accident the affected subjects were careful not to rub their eyes, for rubbing may stimulate the development of illness [2]. Case III was treated with instillation of colloidal silver from the third hour after the accident. Case I was treated with various drugs, but not before the appearance of clinical symptoms. Case II was under clinical observation, but was not treated.

Re-isolation of the virus. Conjunctival washings were taken from the three patients immediately as the first symptoms had been observed (Table I) and from their four coworkers five days later. Virus was isolated from Cases I and II, but neither from Case III nor from the contacts. The failure to isolate virus from Case III can be contributed to the seven-day silver treatment before taking the specimens.

The re-isolated strains were identified by HI and neutralization tests. Both strains proved to be adenovirus type 8. It is noteworthy that the adaptation to tissue culture of re-isolates I and II became complete as soon as in the first (HeLa) and second (Detroit-6 + HeLa) tissue culture passages, respectively, whereas the adaptation of the strains obtained from the 1961–1962 epidemic needed 6–12 passages.

Serological tests were carried out in the paired sera of the three patients, of two family members and of two coworkers (Table II).

At the onset of illness Case I and Case III had no, Case II had very low titre (1 : 4) type 8 neutralizing antibodies. About four weeks later the titres attained the 1 : 16 or 1 : 32 levels. These values though not high, are sufficient to serve as evidence of infection by adenovirus type 8. The in-

Table II

HI and neutralizing antibodies to adenovirus type 8 in the paired sera of the three patients and of the contacts

Subjects	Day of taking blood	Days elapsed since		Antibody titres	
		infection	onset of* illness	HI	Neutr.
Case I	10 Sep., 1962	6	2	8**	—
	8 Oct., 1962	34	30	256	16
Case II	13 Sep., 1962	9	3	32	4
	8 Oct., 1962	34	28	256	32
Case III	13 Sep., 1962	9	3	—	—
	8 Oct., 1962	34	28	128	32
Family member of Case II	13 Sep., 1962	(9)	(3)	—	—
	1 Nov., 1962	(58)	(52)	—	—
Family member of Case III	13 Sep., 1962	(9)	(3)	—	—
	30 Oct., 1962	(56)	(50)	—	—
Coworker I	13 Sep., 1962	Common contacts of cases I—III		—	—
	8 Oct., 1962			—	—
Coworker II	13 Sep., 1962			—	—
	8 Oct., 1962			—	—

* = the day of appearance of conjunctival infiltration

** = reciprocal of serum dilution

— = negative in 1 : 4

crease in titre in Case II was eightfold. Low levels of HI antibodies to adenovirus type 8 were detected in the acute-phase specimens of Cases I and II; in the convalescent specimen of each of the three patients the HI titre was high (1 : 128 or 1 : 256).

Neither neutralizing nor HI antibodies to adenovirus type 8 were found in the convalescent sera of the four contacts, not even two months after the accident, though the patients had not been isolated, but had kept working. They had washed hands more frequently than usual and, naturally, used separate towels. They had separate microscopes or, at least, separate ocular lenses.

Discussion

The aetiology of EKC had been uncertain up to the last few years. A number of different viruses were isolated from patients, even two kinds of virus from the same patient [4—7]. The problem was made difficult by the differences observed in the clinical and epidemiological characteristics of the disease in different parts of the world [2, 4, 8, 9].

At present it is generally accepted that EKC is caused, first of all, by adenovirus type 8 [4, 8, 10, 11]. This view is supported by isolation of the virus from epidemics observed in many parts of the world [2, 8, 12, 13], by extensive serological surveys [7, 14], and artificial infection of volunteers [15]. Furthermore, evidence has been brought that the disease occurring in different countries is the same though its course is influenced by some regional factors [4].

The aetiological study of the 1961—1962 epidemic in Hungary also produced equivocal data at the beginning. A herpesvirus strain was isolated in Debrecen [16] and we isolated type 6 adenovirus. It was only later that we succeeded in isolating seven strains of type 8 adenovirus [1]; the process of adaptation was as slow as in other laboratories [2]. The isolation of virus and the specific increase in the HI titres demonstrated in 94 per cent of the serum pairs tested [1] have proved that the epidemic was caused, first of all, by adenovirus type 8. This statement was confirmed by other investigators [17, 18], and the present observations provide further evidence.

The precise knowledge of the time of infection has made it possible to estimate the incubation period. As shown by Table I, 6—8 days elapsed from the time of infection until the appearance of the first symptoms; the incubation period may be longer with a small dose of virus.

The fact that the closest contacts did not contract the disease, yielded no virus, and did not develop type 8 antibodies, shows that the disease is not highly contagious. None of the four contacts having no specific antibodies developed even inapparent infection. The low contagiousness of the disease is in accordance with the observations [19, 2] that type 8 neutralizing antibodies were detectable in only 4—5 per cent of the healthy subjects tested, whereas a much larger part of the population has antibodies to other types of adenovirus. We found HI antibodies to adenovirus type 8 in a low percentage of healthy subjects [20].

The present data show that out of the two forms of epidemic outlined by JAWETZ [2] the one prevailing in the USA is dominant in Hungary; the susceptibility of the population is not high, the virus penetrates the organism through the tissues of the eyes. Infection does not ensue as a result of a simple contact, it needs direct penetration of a large amount of virus or an adjuvant factor (rubbing, ophthalmological intervention) that promotes the development of corneal lesions. In contrast with this, so-called iatrogenic way of infection, the spontaneous spread of infection dominates in Japan, where, in general, young children are infected by adenovirus type 8, the infection spreads from children to other children and to adults; the epidemic attains a comparatively high incidence. As a result of the high incidence of infection, approximately one-third of the young adults have antibodies to adenovirus type 8 in Japan.

Finally, it is noteworthy that in the present studies as well as in our previous investigations [1] the HI test proved to be as suitable as the neutralization test for the detection of type-specific type 8 antibodies. The former test appeared even more sensitive (Table II). Consequently, the HI test may be used, without carrying out parallel neutralization tests, for the laboratory diagnosis of EKC infections.

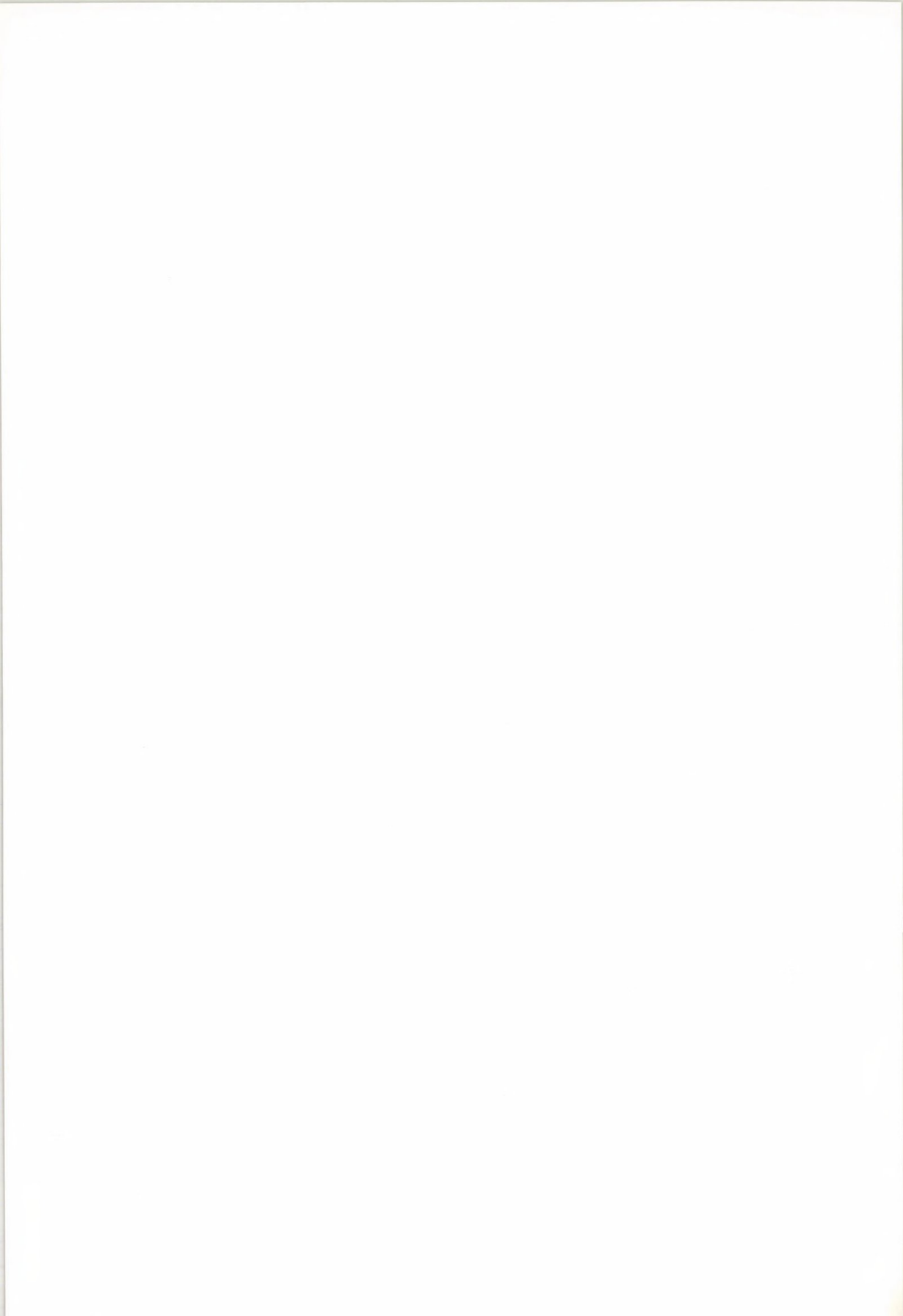
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AETIOLOGIC RELATIONSHIP OF ADENOVIRUS TYPE 8 TO THE EPIDEMIC OF KERATOCONJUNCTIVITIS IN SZEGED

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Summary. Of 52 selected, early and pronounced typical cases of an outbreak of epidemic keratoconjunctivitis in Szeged afflicting over 1500 persons, twelve strains of adenovirus type 8 have been isolated. In these cases a fourfold or greater increase of neutralizing antibodies against the isolated agents was found in seven out of nine pairs of sera tested. From fifteen paired sera of afflicted persons with negative virus isolations a similar rise of antibodies was registered in twelve cases.

The first strain of adenovirus type 8 was isolated from a sporadic though clinically characteristic case of epidemic keratoconjunctivitis (EKC) [1]. Since then, more than 100 isolations of adenovirus type 8 from cases of EKC [2] and inoculations of this agent into volunteers [3] presented convincing evidence of having recovered "at least one of the causes of EKC" [4]. However, most results, apart from those related to the major epidemic in Scotland [5, 6, 7, 8] were based on the investigations of sporadic [9] and endemic cases [10] or on that of minor outbreaks [11]. Only serologic evidences remained to show that the earlier world-wide epidemics of EKC had been caused by adenovirus type 8 [12]. The prevalent role of serotype 8 as the causative agent of large EKC epidemics may be further corroborated by our experience in connection with the 1962 outbreak of the disease in Szeged, part of the epidemic afflicting over 17,000 persons in Hungary.

Materials and methods

Studies were performed in 52 cases of EKC admitted to the Department of Ophthalmology between May 30, and August 8, 1962. These patients were selected from among 1500 afflicted persons on the basis of presenting themselves early after beginning of, or with pronounced, symptoms. All of them were subjected to routine ophthalmologic examination including slit lamp microscopy.

Specimens for virus isolations consisted of eye washings, obtained by dropping repeatedly HANKS' solution supplemented with 200 U Penicillin/ml, 200 μ g Streptomycin/ml, and 50 U mycostatin/ml onto the everted upper and lower palpebral conjunctiva and collecting the fluid covering the conjunctival surface by means of sterile glass capillaries (by capillarity only). Some 0.5—1 ml of the specimens were distributed within 10 minutes between 3—4 stationary tubes of HeLa cell cultures. The maintenance medium was a modified GEY's solution [13], enriched with 5 per cent inactivated rabbit serum and 0.25 per cent lactalbumin hydrolysate; This medium was changed every four to five days. The tubes were inspected for cytopathic changes daily through a period of 28—30 days, and when such changes had become manifest, 0.1 ml of tissue culture fluid was subcultured in HeLa cells. TCID₅₀ of the isolated cytopathic agents were also measured in HeLa cell cultures.

Identification of the isolated agents was performed by neutralization tests, using 100 TCID₅₀ and tenfold dilutions of type-specific rabbit immune sera against types 1, 3, 4 and 5 of adenovirus held previously at 56° C for 30 minutes. In the case of type 7 and 8, a fortyfold dilution of rabbit hyperimmune serum was used.* The virus—serum mixtures were incubated for 2 hours at 37° C and with 0.2 ml of each sample 4 HeLa cell cultures were inoculated. The end results were recorded on the 7th day.

The pathogenic role of the recovered viruses was investigated by comparing the neutralizing antibody titres of paired sera collected on the first day of hospitalization and 3 weeks later. In the cases of successful virus isolations, the viruses, recovered from the respective patients, were used in the neutralization tests, and one strain (Nr 938) of them in cases with negative virus isolations. Neutralization tests were performed with twofold dilutions of the patients' sera, as described above. In the beginning period of the investigations, complement fixing antibodies of paired sera were also determined, by the method of FULTON and DUMBELL [14]. Fluid from tissue cultures of adenovirus type 3 served as antigen.

Results

The 52 cases under study presented themselves with relatively early and pronounced symptoms the most constant of which was an unilateral oedematous swelling of the lids, plica and caruncle with tiny petechiae of the tarsal conjunctiva in the proximity of the lid margins. Some days later the process became mostly bilateral and follicular enlargement of the lower tarsal conjunctiva appeared simultaneously with preauricular adenopathy. The pathognomonic sign of EKC, the keratitis with subepithelial infiltrates, developed some 10 days after the presenting symptoms. From the above signs, petechiae in the proximity of the lid margins occurred in 51, follicular enlargement of the lower fornix in 30, preauricular adenopathy in 24, typical keratitis in 24, pseudomembrane formation in 5 instances out of the 52 cases. Systemic signs (fever, upper respiratory catarrh) were absent in all cases. Iatrogenic spread, in connection with treatment of minor traumatic lesions of the eye, tonometry, etc., might have had a role in 20 cases.

Out of the 52 eye washings of the 52 patients, 13 yielded a cytopathic agent (Table I). Cell degeneration appeared relatively late at first isolation, in two instances on the 26th and 27th day, respectively; in one case, the agent was recovered from the first subpassage only. In further subpassages the cytopathic effect appeared on the 2—3rd day already but the infective titre did not exceed 10³ TCID₅₀/ml.

Of the 13 agents recovered, 12 were neutralized only by rabbit immune serum prepared against adenovirus type 8. Against two strains (No. 911 and 934) protection was complete only when their quantity was 10 TCID₅₀. In contrast to these 12 strains, one agent (No. 956), recovered from a medical student, exerted an early cytopathic effect, reached a high titre and was not neutralized by type 8 antiserum.

Of 10 paired sera available from cases with positive virus isolations, 7 exhibited a greater than fourfold rise in the titre of neutralizing antibodies

* Kindly supplied by Dr. D. A. J. TYRRELL, *Common Cold Research Unit, Salisbury.*

Table I

*Data concerning clinical features, results of virus isolations
and antibody titres of paired sera in 13 positive cases of virus isolations*

Number of cases	Age (years), sex	Clinical symptoms*	The day of sample taking	Day of appearance of cytopathic effect	Type of rabbit serum neutralizing virus (100 TCID ₅₀)	Serological tests with paired sera			
						neutralizing antibody titres ³		complement fixing antibody titres ³	
						acute	convalescent	acute	convalescent
905	18 ♀	p, f, a	1st	21st ¹	8	<4	16	<2	4
907	62 ♂	p, k	10th	26th	8	<4	8	<2	8
910	32 ♂	p, f, k	1st	18th	8	<4	64	<2	4
911	50 ♂	p, a	2nd	22nd	8 ²	<4	32	4	16
917	31 ♂	p, f	5th	18th	8	<4	32	<2	8
934	2 ♀	p, a		16th	8 ²				
936	41 ♀	p, f, ps	2nd	27th	8	<4	32	<2	8
938	36 ♂	p, ps, k	3rd	10th	8	<4	16	<2	4
954	54 ♂	p, a	2nd	23rd	8	4	16		
956	21 ♀	p, a	1st	6th	none of 1, 3, 4, 5, 7 and 8	4	8		
962	10 ♀	p, k	4th	27th	8	4	4		
967	55 ♀	p, k	13th	14th	8				
968	23 ♂	p, f, ps	3rd	7th	8				

* Clinical symptoms: p: conjunctival petechiae near the margin of swollen lids; f: follicular enlargement of lower tarsal conjunctiva; ps: conjunctival pseudomembranes; a: preauricular adenopathy; k: subepithelial infiltrates on the cornea.

¹ Isolated from 1st subpassage after blind passage made on 18th day after inoculation of eye washing fluid.

² Only 10 TCID₅₀ of the isolated agents were consistently neutralized.

³ Reciprocal values of serum dilutions.

during convalescence. In the remaining 3 cases (No. 907, 956 and 962), the increase of neutralizing antibodies was less significant. In case 907, however, a more than fourfold rise in titre was found in the complement fixation test, and from case 956 an adenovirus differing from serotype 8 was isolated.

A more than fourfold rise of neutralizing antibodies against one (No. 938) of the isolated type 8 strains was found also in 12 out of the tested 15 cases with negative virus isolations. Of the remaining 3 cases without any immune response, one suffering from endogenous uveitis was under prednisolone treatment, another recovered after 3 days from his mild disease, having already had neutralizing antibodies in the acute phase; in the third case the lack of neutralizing antibody formation might have been responsible for a serious relapse.

Discussion

Of the 52 cases under study, the data of 12 patients have furnished strong evidence of an aetiological relationship of type 8 adenovirus to the extensive 1962 epidemic of keratoconjunctivitis in Szeged. All were cases with typical symptoms of EKC, similar to those described in the great American outbreaks 1941—43 [15, 16]. From all 12 cases, adenovirus type 8 was isolated. Among the 9 paired sera available, in 7 a fourfold or greater increase of neutralizing antibody titre against the isolated strain, and in a further case that of complement fixing antibodies, was found during the convalescence, suggesting the pathogenic role of the isolated agents.

The fourfold or greater increase of neutralizing antibodies against adenovirus type 8 in 12 of 15 paired sera of patients yielding no cytopathic agents, was some evidence of the aetiological role of serotype 8 although not quite conclusive, as human adenovirus infections may, though inconsistently, induce immunologic responses against heterologous serotypes.

Of 52 attempted virus isolations 39 yielded no cytopathic agent, and from one case an adenovirus differing from serotype 8 was isolated, against which no significant rise of neutralizing antibodies occurred during the convalescence. These negative results do not preclude, however, the aetiological role of serotype 8, as all reports on virus isolation procedures from cases of EKC share this low rate of positive results. Even other serotypes present, but not responsible for the disease, were recovered from cases caused by adenovirus type 8, as suggested by the neutralization tests of paired sera [5, 6]. All these negative findings may be explained on the basis of the comparatively low rate of infective particles of serotype 8 present [2], as evidenced by the late manifestation of cytopathic changes in tissue cultures and the low titres of the recovered agents. The 39 cases with negative virus isolation were characterized by a somewhat milder course (mean of neutralizing antibody titres during convalescence in the 15 cases tested 1 : 11.2 as compared to 1 : 22.8 in the cases yielding type 8 adenovirus) and a later availability of specimens for virus isolation (mean, 6.4 days after onset of the disease, as compared to 3.9 day in the cases of positive virus isolation), both circumstances further decreasing infectivity and the chances of virus isolation. On the other hand, young children's washings (cases 934 and 967) manifested a comparatively early cytopathic effect like those of the most serious cases (No. 938 and 968), yielding larger amounts of infective virus apt to spread without any iatrogenic transfer.

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KONGRESS DER UNGARISCHEN MIKROBIOLOGISCHEN GESELLSCHAFT

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ZUSAMMENFASSUNGEN

BAKTERIENGENETIK

G. IVÁNOVICS, K. CSISZÁR

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TRANSDUKTIONSVERSUCHE MIT DEN AUXOTROPHEN VON *B. SUBTILIS*

Von einem Teil der temperierten *Subtilis*-Phagen, die auf Grund nicht nur der plasmorphologischen, sondern auch der Antigenstruktur identisch erscheinen, werden von Prototrophen verschiedene Loci auf einzelne oder doppelte Auxotrophie übertragen. Das Transduktionsvermögen der einzelnen Phagen zeigt ein unterschiedliches Ausmaß. Durch Studium der 6 verschiedenen Histidin-Indol-Doppelauxotrophen von ZAMENHOF und Mitarbeitern wurden die eigenen Ergebnisse mit der von genannten Autoren mit DNS-Transformation festgestellten Genstruktur verglichen. Bei der Transduktion konnte die Position der einzelnen Histidin-Loci nicht am selben Ort lokalisiert werden. Die feinere Struktur der an Histidin-Indol gekoppelten Loci von *B. subtilis* muß daher in weiteren Untersuchungen klargestellt werden.

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ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNG DES KP (KILLER-PRINCIPLE) VOM *B. MEGATERIUM*-STAMM 337

Die elektronenmikroskopische Untersuchung des Stammes Nr. 337 von *B. megaterium* ergab eine bisher unbekannte Zellstruktur. An der Oberfläche der sehr leicht zerfallenden Zellen sind sich halbkugelig vorwölbende »tumorartige« Formationen zu sehen, deren Zentrum »ulzeriert«. In den Schnitten ist die Zellwand der Bakterien an zahlreichen Stellen vom Zytoplasma desquamiert und dementsprechend vakuolisiert. Neben dieser eigenartigen Struktur sieht man um die Bakterien kugelige Gebilde mit einem Durchmesser von 54 m μ . Diese Formationen gleichen jedoch nicht den üblichen inkompletten Phagpartikelchen, so daß ihre Natur unklar ist. Untersuchungen zwecks Identifikation dieser Gebilde mit der für die KP-Wirkung verantwortlichen Substanz sind im Gange.

G. IVÁNOVICS, E. MARJAY

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ÜBER EINE NEUE BACTERIOCINARTIGE SUBSTANZ DES *B. MEGATERIUM*

Der *B. megaterium*-Stamm Nr. 337 setzt in seiner sporentragenden und asporogenen Form eine eigenartige bacteriocinartige Substanz frei, die bakterizid auf die phagempfindlichen *B. megaterium*-Stämme wirkt. Im Gegensatz zu Megacin sind die phagresistenten Stämme der Substanz gegenüber refraktär. Dieses mit dem Namen KP bezeichnete Prinzip unterscheidet sich in seiner Entstehung und in den anderen Eigenschaften scharf von Megacin. Eine seiner charakteristischen Eigentümlichkeiten besteht darin, daß das KP in der präparativen Ultrazentrifuge sedimentiert werden kann. Die anderen Eigenschaften dieses partikulierten Prinzips wurden gleichfalls besprochen.

L. ALFÖLDY

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DIE REGULATION DER SYNTHESE VON *E. COLI*-RIBONUKLEINSÄURE AUF GRUND GENETISCHER VERSUCHE

Das Ausmaß der RNS-Synthese in *E. coli* hängt von der Eiweißbildung ab. Auxotrophe Stämme synthetisieren RNS nur in Anwesenheit der erforderlichen Aminosäure, nach deren Entzug die RNS-Synthese sogleich zum Stillstand kommt.

BOREK und Mitarbeiter fanden einen Stamm, der auch nach Aminosäure-entzug zur RNS-Synthese fähig ist, während STENT und BRENNER nachwiesen, daß sich ein großer Teil der bei den bakteriumgenetischen Versuchen benutzten Hfr-Stämme ähnlich verhält und diese Eigenschaft durch Konjugation auch auf andere Stämme übertragen werden kann.

Im Rahmen eigener Versuche bestimmten wir mit Hilfe der Konjugationsmethode genau den Platz des die RNS-Synthese regulierenden Locus (RC-Locus) auf der Chromosomenkarte von *E. coli* und vermochten zu bestätigen, daß sich dieser nahe dem Streptomycin-Locus befindet.

Durch die im RC-Locus eintretende Mutation wird die Regulation der RNS-Synthese des Bakteriums von der strengen Aminosäurekontrolle befreit (Relaxation). Durch die Anwesenheit einzelner Aminosäuren, die imstande sind, das Wachstum des Stammes zu hemmen, werden diese Stämme gleichzeitig empfindlicher.

Es wird versucht, aus den beim Studium des Relaxationszustandes gewonnenen Ergebnissen einige Rückschlüsse auf den Regulationsmechanismus der RNS-Synthese zu ziehen.

CHEMOTHERAPIE

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VERSUCHE ZUR CHEMOTHERAPEUTISCHEN BEEINFLUSSUNG DER TRICHOMONIASIS

Verff. stellten 35 organische Basen (bzw. ihre Salze), 17 Arylarsinsäure-Derivate und 3 semiorganische Quecksilberverbindungen her. Die trichomonostatische Wirkung dieser Verbindungen sowie die von 22 (zum Teil aus dem Pharmakologischen Institut der Medizinischen Universität Debrecen stammenden) Antibiotika wurde *in vitro* untersucht.

Die Wirkung der folgenden Verbindungen erschien so günstig, daß ihre klinische Erprobung zur Lokalbehandlung der Trichomoniasis empfohlen wird:

1. Poly-(1,8-diaza-undecan) hydrobromid,
2. Poly-(1,8-diaza-10-hydroxy-undecan) hydrochlorid,
3. S-n-Dodecyl-isothiuronium-jodid,
4. N¹,N⁵-Di(4'-chlor-phenyl)-diguanidin hydrochlorid,
5. 3-Acetyl-amino-4-hydroxy-phenyl-arseno-di-(thioglycolsäure),
6. S-(2-Hydroxy-5-methyl-phenyl-mercuri)-thioglycolsäure (Netropsin).

Mit Poly-(1,8-diaza-10-hydroxy-undecan)-hydrochlorid, S-n-Dodecyl-isothiuronium-jodid und e-Acetyl-amino-4-hydroxy-phenyl-arseno-di-(thioglycolsäure) hat Chefarzt Dr. T. Kovács an umfangreichem Trichomoniasis-Krankematerial therapeutische Untersuchungen durchgeführt und im Falle lokaler Anwendung mit den ersten beiden recht günstige (Heilung in 85 bzw. 68% der Fälle), mit dem letzteren hingegen mittelmäßige Ergebnisse (50%ige Heilung) erzielt.

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VERSUCHE MIT EINIGEN AUF RESISTENTE STAPHYLOKOKKEN WIRKENDEN TETRACYCLIN-DERIVATEN

Nach Literaturangaben ist der Lipoidgehalt in der Zellwand resistenter Mikroorganismen im Vergleich zur empfindlichen Variante vermehrt. Wahrscheinlich wird das Eindringen der Antibiotika in die Zelle durch diese Lipoidhülle verhindert. Im Verlauf der Untersuchung wurden an oberflächenaktive Substanzen gekoppelte Tetracyclin- und Oxytetracyclin-Derivate hergestellt, und zwar von der Voraussetzung ausgehend, daß die Derivate infolge ihrer

stärkeren Oberflächenaktivität durch die Lipoidbarriere der resistenten Zellen zu dringen vermögen. Von den synthetisierten Verbindungen wurde die Tetracyclin-Resistenz der untersuchten Staphylokokken- (und *E. coli*-) Stämme den Erwartungen entsprechend mehr oder minder herabgesetzt. Die Resistenzverminderung stand im Verhältnis zum Ausmaß der Resistenz des untersuchten Stammes. Bei ausgeprägter Tetracyclin-Resistenz (10–40 $\mu\text{g/ml}$) war die resistenzverringende Wirkung des aktivsten Derivates bei der überwiegenden Mehrheit der untersuchten Stämme 8–10fach, im Falle mäßiger Resistenz 2–4fach, während auf empfindliche Zellen das Derivat in derselben Konzentration wirkte wie die Grundverbindung. Das Wirkungsoptimum kam bei der an verschiedene Ringe substituierten Seitenkette mit 12 Kohlenatomen zustande. Die zwischen dem Tetracyclinmolekül und der Seitenkette entstandene Bindung erwies sich bei der Untersuchung des Infrarotspektrums des Derivates als sehr stabil. Bei oraler Verabreichung betrug der LD_{50} -Wert von Tetracyclin 2500 mg/kg, der LD_{50} -Wert des Derivates mehr als 20 000 mg/kg. Nach Reihenuntersuchungen an Kaninchen erreichte die antibiotische Blutaktivität nach oraler Darreichung das Maximum beim Derivat etwas langsamer als bei Tetracyclin; demgegenüber war der Wert nach 8 Stunden praktisch unverändert, während zu diesem Zeitpunkt bereits etwa 20% der Grundverbindung ausgeschieden waren. Die klinische Erprobung des Derivates, das sich am wirksamsten erwies, ist im Gange.

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VERGLEICHENDE UNTERSUCHUNG VON BAKTERIOSTATISCHEN
UND BAKTERIZIDEN WIRKUNGEN
NACH DER REPLICA-GRADIENTPLATE-METHODE

Unter Zusammenfassung und Modifikation der Methoden von SZYBALSKI und SZTEITFELD wurden nach dem Replica-gradient-plate-Verfahren die bakteriostatischen und bakteriziden Wirkungen antibakterieller Substanzen auf die Krankheitserreger pyogener Prozesse und auf *Dyspepsiecoli* Stämme untersucht.

Wie die Empfindlichkeitsuntersuchungen ergaben, gesellt sich im Rahmen der angewendeten Konzentrationen zur bakteriostatischen Wirkung der allein angewendeten Substanzen sowie ihrer Gemische in mehr als der Hälfte der Fälle bei Anwesenheit gleicher oder höherer Wirkstoffmengen auch ein bakteriozider Effekt.

Es wird darauf hingewiesen, daß es wichtig sei, bei der Aufnahme des Antibiotogramms in den sich hinziehenden Krankheitsfällen mit bakterieller

Ätiologie auch die bakteriziden Wirkungen zu ermitteln. Die auf diese Weise festgestellten bakteriziden Substanzen sind als die therapeutisch günstigsten zu betrachten und in der Behandlung zu verwenden.

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(Seruminstitut Humán, Budapest)

NEUE BEFUNDE ÜBER DIE SYNERGISTISCHE
UND ANTAGONISTISCHE WIRKUNG DER ANTIBIOTIKA

Bei der Untersuchung der synergistischen und antagonistischen Wirkung von 12 Antibiotika (Penicillin, Streptomycin, Chloramphenicol, Aureomycin, Terramycin, Polymyxin B, Neomycin, Erythromycin, Oleandomycin, Vakomycin, Viomycin, Kanamycin) wurde festgestellt, daß nur einige Antibiotikumkombinationen unabhängig von den Bakterienstämmen synergistisch wirken, z. B. Penicillin, Streptomycin, Strepto-Neomycin, Penicillin-Erythromycin, Neomycin-Polymyxin, oder sicher als antagonistisch wirkend angesehen werden können, wie Chloramphenicol-Streptomycin, Penicillin-Aureomycin, Oleandomycin-Vakomycin. Zur Vermeidung der raschen Resistenzentwicklung empfiehlt sich bei den anderen Substanzen die Untersuchung der Wechselwirkung nach der Verdünnungsmethode. Bei der Kombinationsbehandlung mit ungenügenden Mengen reicht nämlich der sinkende Blutspiegel in den Dosierungsintervallen vermutlich aus, um die Resistenzentwicklung zu begünstigen. Insbesondere bei denjenigen resistenten Bakterien lassen sich günstige Ergebnisse mit der Kombinationsbehandlung erzielen, deren Vermehrung auf antibiotikumhaltigen Nährboden nicht dasselbe Tempo zeigt wie die der Kontrolle, die vielmehr die Keimzahl der Kontrollkultur erst nach einer längeren Latenzperiode erreichen.

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ÜBER DIE FEHLERQUELLEN BEI DER BESTIMMUNG
DER ARZNEIRESISTENZ DER TUBERKELBAKTERIEN

Bei der Untersuchung der Arzneiempfindlichkeit von Tuberkelbakterien ist häufig ein Unterschied in der klinisch und bakteriologisch feststellbaren Resistenz zu beobachten. Beim Studium der Ursachen der bei der Resistenzbestimmung wahrgenommenen Schwankungen untersuchten wir die Wirkung der Lagphase, der Generationszeit der Bakterien und der bei der Kolonien-

zählung festgestellten Streuung auf die Untersuchungsergebnisse. Hierbei gelangten wir zu der Überzeugung, daß die erwähnten Faktoren bei der Arzneipflichtigkeitsuntersuchung einen wesentlichen Einfluß auf die Zusammensetzung der Bakterienpopulation in einem gegebenen Zeitpunkt ausüben.

BAKTERIOLOGISCHE BIOCHEMIE

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DIE NUKLEINSÄURE-ZUSAMMENSETZUNG VON STREPTOMYCES GRISEUS-STÄMMEN

In verschiedenen Wachstumsphasen wurde die Phosphorverteilung in *Streptomyces griseus*-Stämmen untersucht. Wir verglichen den Nukleinsäurestoffwechsel eines streptomycinerzeugenden *Str. griseus*-Stammes mit dem der Streptomycin nicht produzierenden Mutante in verschiedenen alten Kulturen. In ihren Wachstumsphasen und in der Dauer der Phasen zeigen diese beiden Stämme gewisse Unterschiede. Wir suchten nach einen Zusammenhang zwischen den Wachstumsphasen und der Nukleinsäurezusammensetzung dieser Mikroorganismen.

Die Fraktionierung der gewaschenen Mycel-Suspension wurde nach dem von SCHNEIDER modifizierten SCHMIDT—TANNHAUSERSchen Verfahren (SCHNEIDER 1946) vorgenommen. Den Phosphorgehalt der einzelnen Fraktionen bestimmten wir nach ROBINSON—MARTLAND.

Die Untersuchungen ergaben folgendes: 1. In Übereinstimmung mit den Literaturangaben zeigt der auf die Trockensubstanz bezogene Gesamtnukleinsäurewert bei den verschiedenen Fermentationen hochgradige Schwankungen. 2. Am höchsten ist der Nukleinsäurewert in jungen Kulturen (24 Stunden); in den späteren Fermentationsstadien wird er nach und nach niedriger. 3. Das RNA/DNA-Verhältnis verschiebt sich in älteren Kulturen (96 Stunden) mehr und mehr zugunsten von DNA. 4. Die Heterogenität und das wechselnde Verhältnis der vegetativen und reproduktiven Elemente in den Kulturen erklären die hochgradigen Schwankungen des Nukleinsäuregehalts im Mycel.

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UNTERSUCHUNG DES LEBENSZYKLUS VON STREPTOMYCIN ERZEUGENDEN UND NICHT ERZEUGENDEN *STR. GRISEUS*-STÄMMEN IN TIEFKULTUREN

Der Verlauf des Lebenszyklus eines Streptomycin erzeugenden und eines Streptomycin nicht erzeugenden *Str. griseus*-Stammes wurde in Tiefkulturen auf synthetischem Nährboden untersucht. Es konnten bedeutende morphologische und histochemische Unterschiede im Verlauf des Lebenszyklus der beiden Stämme festgestellt werden.

Der Lebenszyklus des Streptomycin nicht erzeugenden Stammes ist kurz, komplett und endet mit der Bildung einer großen Sporenmenge. Beim kurzen Lebenszyklus ist mit geringer Vergrößerung eine charakteristische morphologische Formation, der sog. »Knoten«, zu beobachten. Bei Immersionsvergrößerung stellen die charakteristischen Wandveränderungen der reproduktiven Mycelien die augenfälligste morphologische Erscheinung dar. Vor der Bildung der Sporenform findet bei jeder Stufe der bezeichnende Umbau der PAS-positiven Polysaccharide der reproduktiven Mycelien statt.

Der Lebenszyklus des streptomycinerzeugenden Stammes ist unter denselben Bedingungen nicht komplett, d. h. er gelangt nicht bis zur Sporenbildung. Aus den morphologischen und histochemischen Untersuchungen darf auf eine Blockierung des Lebenszyklusprozesses geschlossen werden.

Den für den kurzen Lebenszyklus verantwortlichen Faktor vermochten wir in der Fermentbrühe des Streptomycins nicht erzeugenden Stammes nachzuweisen.

Unter Wirkung dieses Faktors wird der Lebenszyklus des streptomycin-erzeugenden Stammes kürzer, seine Differenzierung tritt rasch ein.

Der den kurzen Lebenszyklus hervorrufende Faktor wirkt spezifisch, d. h. nur auf *Str. griseus*-Stämme. Der Faktor ist thermolabil und diffundiert nicht durch die Cellophanmembran.

Das Isolierungsverfahren des Faktors und die chemische Natur der partiell gereinigten Substanz wurden besprochen.

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UNTERSUCHUNG DER ZELLWANDZUSAMMENSETZUNG
IN *STREPTOMYCES GRISEUS*-STÄMMEN

Der Kohlenhydratgehalt in der Zellwand von zwei *Str. griseus*-Stämmen wurde untersucht. Der eine (Nr. 52—1) erzeugte Streptomycin, der andere (Nr. 45 H) nicht. Wir führten quantitative und qualitative Kohlenhydratbestimmungen durch. Beide Stämme enthielten Hexose und Pentose. Bei den papierchromatographischen Untersuchungen erwies sich die Pentose als Arabinose. Diese Tatsache steht im Gegensatz zu den Ergebnissen von ROMANO und SOHLER (J. Bact. 72, 865, 1956), denn nach ihren Angaben soll die Zellwand der *Nocardia* Pentose enthalten, die der *Streptomyces* aber nicht.

Fernerhin untersuchten wir, ob in der Kohlenhydratzusammensetzung der Zellwand des streptomycinerzeugenden (52—1) und des Streptomycin nicht produzierenden Stammes (45 H) ein Unterschied festgestellt werden könne.

In der Kohlenhydratzusammensetzung der Zellwand des streptomycinerzeugenden und Streptomycin nicht produzierenden Stammes fanden wir einen quantitativen Unterschied.

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STRAHLENEMPFFINDLICHKEITSUNTERSUCHUNGEN
AN *E. COLI* B-KULTUREN

Verff. untersuchten die Strahlenempfindlichkeit von ventilierten *E. coli* B-Kulturen in verschiedenen Entwicklungsphasen.

Wie festgestellt wurde, ist die Kultur der ionisierenden Strahlung gegenüber in der Übergangsphase zwischen der Inkubations- und Logphase am empfindlichsten.

Auf Grund der Versuchsergebnisse wurden theoretische Rückschlüsse auf den Stoffwechsel der in den verschiedenen Züchtungsphasen befindlichen Mikroorganismen gezogen. In der Praxis sollen die Erfahrungen bei der Wertbestimmung der Strahlenschutzstoffe verwertet werden.

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WACHSTUM EINES STREPTOMYCINBEDÜRFTIGEN
E. COLI-STAMMES AUF STREPTOMYCINFREIEM NÄHRBODEN

Bei dem streptomycinbedürftigen *E. coli*-Stamm Sd-4—73 wurde unter Anwendung der Agardiffusionsmethode um das ins Loch gegebene Methanol im Falle einer gewissen Inokulumgröße auf streptomycinfreiem Nährboden eine Wachstumszone beobachtet. Hiervon ausgehend, untersuchten wir die Quantität und Qualität des im streptomycinfreien Nährboden vor sich gehenden Residualwachstums unter verschiedenen Bedingungen.

Wie aus Literaturangaben bekannt, ist die Zahl der Residualteilungen von der Konzentration des Inokulums abhängig; bei einer gewissen Inokulumgröße entstehen vielkernige Fäden nach den Teilungen.

Diese Erscheinung konnte mit folgenden Faktoren beeinflusst werden: Von höherer anorganischer P- und Saccharosekonzentration sowie niedrigerer Temperatur wird die Residualteilung in der Bouillon und im ammoniumsulfathaltigen synthetischen Nährboden stimuliert. Pyrimidin-Basen steigern im Gegensatz dazu das Übergewicht der Fäden, indessen tritt diese Wirkung nur in dem mit Casamin angereicherten synthetischen Nährboden wesentlich zutage. Purinbasen wirken nicht bzw. weniger. Unter Wirkung von Methanol (und einigen anderen Alkoholen) wächst die Zahl der Teilungen in der Bouillon auf das Mehrfache, was die eingangs erwähnte Beobachtung erklärt. Auf synthetischem Nährboden aber wird von Methanol nur die Fadenbildung gehemmt.

Diese Wahrnehmungen deuten wir folgendermaßen: Streptomycin ist zur Wandsynthese erforderlich. Wenn es fehlt, so wird das Gleichgewicht der Wand- und der Plasmasynthese verändert. Von obigen Faktoren wird dieses Gleichgewicht in einer für die Teilung günstigen oder ungünstigen Richtung beeinflusst.

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DIE VERÄNDERUNG DER PHOSPHORVERTEILUNG
IN DER B-ZELLFRAKTION VON *E. COLI* UNTER WIRKUNG
IONISIERENDER STRAHLUNG

Der Phosphorgehalt der Zellfraktionen in der *E. coli* B-Kultur wurde nach Bestrahlung mit hohen Rtg-Dosen untersucht. Bei der Fraktionierung kam die von SCHNEIDER modifizierte SCHMIDT—TANNHAUSERSche Methode zur Anwendung. Nach der Bestrahlung war der Nukleinsäure-Phosphorgehalt

vermindert und zugleich der säurelösliche Phosphorgehalt vermehrt. Deshalb erfolgte die eingehendere Untersuchung einzelner Bestandteile der säurelöslichen Phosphorfraktion nach LE PAGE.

Aus den Ergebnissen wurden Schlußfolgerungen auf die Wirkung der ionisierenden Strahlung auf die Nukleinsäuren unter in vivo-Bedingungen gezogen.

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DIE REGULATION DES VALIN- UND ISOLEUCINSTOFFWECHSELS
BEI *PSEUDOMONAS AERUGINOSA*

Zugleich mit dem Wachstum wurde die an der Biosynthese von Valin und Isoleucin teilnehmende Azetomilchsäure bildende Enzymmenge bestimmt. In *Ps. aeruginosa*-Zellen bzw. in den Extrakten erschlossener Zellen ermittelten wir die Aktivität dieses Enzyms und der Dioxysäuredehydrase.

Nach den Ergebnissen beginnt der auf das Bakteriengewicht bezogene Enzymspiegel noch vor der Logphase zu steigen; in der 3. Stunde erreicht das Wachstum den Höhepunkt und fällt noch während der Logphase auf das Ausgangsniveau zurück. Die Größe des Maximums wird von einem Endprodukt der Biosynthese, von Valin, herabgesetzt, vom anderen Endprodukt, Isoleucin, gesteigert. Unter Wirkung von Isoleucin bleibt der Enzymspiegel bis zum Ende der Züchtung höher. In der Dioxysäuredehydrase-Aktivität sind unter Wirkung von Valin und Isoleucin ähnliche Aktivitätsveränderungen zu beobachten.

Die gewonnenen Resultate lassen sich mit der gegenwärtig anerkannten Theorie der Enzymregulation gut erklären: die Menge der an der Valin- und Isoleucinsynthese teilnehmenden Enzyme wird von der intrazellulären Valin- und Isoleucinkonzentration reguliert. Bei *Ps. aeruginosa* unterdrückt Valin die Synthese dieser Enzyme, und diese Repression wird von Isoleucin aufgehoben, das eine sog. »Endproduktinduktion« verursacht.

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DIE ENZYMEIWEISS-SYNTHESE UND REGULATION
DES *B. CEREUS*-STAMMES 569 DURCH DEN STOFFWECHSEL

Im Rahmen der Versuche wurde die Enzymerzeugung des Penicillinase induktiv produzierenden *B. cereus*-Stammes 569 und die Regulation des Mechanismus der Penicillinwirkung auf Pepton-Hefeextrakt und synthetischem

Nährboden untersucht. Es konnte folgendes festgestellt werden: 1. Der Mechanismus der mit Penicillin induzierten Lysis ist mit der durch »Stoffwechselstörung« zustande gekommenen Lysis nicht identisch. 2. Die mit Penicillin induzierte Lysis setzt nach der totalen Penicillinhydrolyse ein; während der Lysis nimmt die Erzeugung bzw. Freisetzung der Penicillinase gleichmäßig zu. 3. Die Penicillinaseproduktion wird von der Zusammensetzung des Nährbodens reguliert: vom Hefeextrakt wird sie in geringem Maße, von der Glukose hochgradig gehemmt. 4. Die mit Penicillin induzierte Lysis wird vom Hefeextrakt und von den zweiwertigen Kationen folgendermaßen reguliert: a) Der im Hefeextrakt enthaltene »lytische Faktor« ist für die mit Penicillin induzierte Lysis der Zellen verantwortlich. Den lytischen Faktor haben wir isoliert und beschrieben. b) Die Lysis wird von Zn^{++} und Mn^{++} katalysiert. Wir bestimmten auch die quantitativen Verhältnisse einiger Faktoren dieses Regulationsmechanismus.

ALLGEMEINE MIKROBIOLOGIE

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LICHT- UND ELEKTRONENMIKROSKOPISCHE UNTERSUCHUNGEN ÜBER DIE DURCH VERSCHIEDENE EINWIRKUNGEN ENTSTANDENEN SPHÄROPLASTE VON *SALMONELLA TYPHI-SUIS* VAR. VOLDAGSEN

Bei der licht- und elektronenmikroskopischen Untersuchung (in ultradünnen Schnitten) der mit Penicillin, Glycin sowie mit Lithiumchlorid gestalteten Sphäroplasten konnten deutliche Unterschiede im Entwicklungsmechanismus, in den biologischen Eigenschaften und in der Ultrastruktur der einzelnen Sphäroplastarten festgestellt werden.

So scheint als wesentlicher Unterschied erwähnenswert, daß sich im flüssigen Nährboden die entstandenen Penicillin- und Glycin-Sphäroplaste nicht vermehren, während sich die Lithiumchlorid-Sphäroplaste durch Teilung vermehren. Das Penicillin-Sphäroplast wächst daher z. B. nicht selten zu einer Größe von 10–15 μ , weil seine kontinuierliche Synthese nicht durch Teilung unterbrochen wird. Von Penicillin wird der Teilungsmechanismus sogleich zum Stillstand gebracht, von Glycin und von Lithiumchlorid — in der für die Sphäroplastbildung optimalen Konzentration — jedoch nicht. Die Densität der mit Penicillin behandelten Kulturen läßt daher nach einer gewissen Zeit nach, während die der anderen beiden stark zunimmt. Das Penicillin- und das Glycin-Sphäroplast verlieren ihr Bewegungsvermögen, hingegen zeigt das Lithiumchlorid-Sphäroplast lebhaftige Bewegung.

Befinden sich die Bakterien unter Wirkung der schädigenden Substanz auf festem Nährboden und unter anaeroben Bedingungen, so wird der Teilungsmechanismus auch von Penicillin weniger gehemmt, und die Bakterien vermehren sich in der Regel in zwei, morphologisch scharf unterschiedlichen Phasen: in langen Formen und in Sphäroplastformen. Diese beiden morphologischen Phasen treten auch in den Glycin- und Lithiumchlorid-Kulturen in Erscheinung.

Die Untersuchungen erstreckten sich fernerhin auf die Messung der osmotischen Stabilität der Sphäroplaste, die auch im Zusammenhang mit der elektronenmikroskopischen präparativen Arbeit eine sehr wesentliche Frage darstellt. Von den Sphäroplasten sind in der Literatur nur wenige Aufnahmen ultradünner Schnitte veröffentlicht worden, und deren Qualität ist nicht zufriedenstellend. Dies beruht darauf, daß die auf osmotische und oxydative Wirkungen außerordentlich empfindlich reagierenden Zellen während des Fixierungs- und Einbettungsprozesses schwere Schädigungen erleiden.

Es ist uns gelungen, eine Fixierungs- und Einbettungsmethode auszuarbeiten, nach der die Sphäroplaste unbeschädigt eingebettet werden können. In den auf diese Weise gewonnenen ultradünnen Schnitten hatten wir Gelegenheit, die Zellwand, die Plasmamembran, das Plasma und die Kernsubstanz, die Vakuolen, die »Graukörper«, die mitochondrienartigen Formationen sowie eigenartige Teilungserscheinungen zu beobachten und zu messen. Diese Beobachtungen sowie die Ergebnisse von Versuchen zur Gestaltung der L-Form werden besprochen.

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EXPERIMENTELLE *E. COLI* 0124 : B 17-KERATOCONJUNCTIVITIS

Bei Meerschweinchen entwickelt sich regelmäßig Keratoconjunctivitis nach konjunktivaler Infektion mit virulenten *E. coli* 0124 : B 17-Kulturen. Andere Colistämme — die enteropathogenen einbegriffen — wirken auf das Meerschweinchenauge nicht pathogen. Die klinischen, histologischen, bakteriologischen und serologischen Eigentümlichkeiten der Coli-Keratoconjunctivitis entsprechen den im Verlauf der Keratoconjunctivitis shigellosa eintretenden Veränderungen. Durch die nach Heilung vorgenommenen wiederholten Infektionen entwickelt sich Resistenz, die jedoch den Kriterien der artspezifischen Immunität nicht entspricht, da wechselseitige Kreuzimmunität zwischen dem Krankheitserreger und den Shigella-Typen festgestellt werden kann. In seinen anderen Eigenheiten zeigt der *E. coli* 0124 : B 17-Stamm gleichfalls Verwandtschaft mit der Shigella-Hauptgruppe (sein somatisches Antigen ist mit 3 *Sh. dysenteriae*-Typen identisch, Laktose fermentiert er

höchstens spät, auf DC bildet er shigellaartige Kolonien usw.). Auf Grund dieser Tatsachen ist der fragliche *E. coli*-Typus in systematologischer Beziehung als eine Übergangseinheit zwischen der Shigella- und *E. coli*-Hauptgruppe zu betrachten.

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UNTERSUCHUNG DER INFEKTIONSDYNAMIK IM BAKTERIENINFIZIERTEN TABAKSBLATT

Das Studium und Verständnis der Pathogenität bzw. die Wechselbeziehungen des Wirtsparasiten rücken immer mehr in den Vordergrund der Phytopathologie.

Aus dem Vermehrungsprozeß der in das Tabaksblatt infiltrierten Bakterien schlossen wir auf die in der Pflanze vor sich gehenden Abwehrprozesse. Die in die Pflanze eingeführten Bakterien wirken einerseits als pathogene Agenzien, andererseits zeigt ihr Vermehrungsprozeß indikatorartig die Abwehrreaktionen der Pflanze an.

Die Versuche ergaben, daß die Zellzahl des den kongenialen Wirt infizierenden pathogenen Agens nach ähnlichen Gesetzmäßigkeiten zunimmt, als ob sie sich im Nährboden vermehren würde. Daraus darf geschlossen werden, daß eine die Vermehrung des Krankheitserregers hemmende Reaktion der Pflanze nicht erscheint. — Sofern ein Erreger nicht seine Wirtspflanze infiziert, wird von der Initialvermehrung des Bakteriums eine hypersensitive Reaktion der Pflanze induziert. Diese postinfektiöse Reaktion manifestiert sich einerseits in der Nekrose einiger Wirtszellen, andererseits in der Hemmung der Bakterienvermehrung.

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MORPHOLOGISCHE KOLONIEVARIANTEN DER *SHIGELLA FLEXNERI*-TYPEN

In im schräg durchfallenden Licht untersuchten Agarplattenkulturen von *Sh. flexneri* isolierten wir morphologische Varianten, die im auffallenden Licht nicht differenziert werden können. Zumeist handelt es sich um fixe Varianten, deren Antigen sich nicht nachweisbar verändert hat, so daß sie nicht zu den R-Modifikationen gerechnet werden können; ebenso kann die Antigendegradation in X- oder Y-Richtung und die sog. Omega-Variation ausgeschlossen werden. Auf die Meerschweinchen-Konjunktiva wirken die

optisch homogenen Varianten konsequent virulent, die meisten inhomogenen Varianten avirulent. Die Mäusevirulenz war nur bei der Variante »konzentrisch 2« schwächer. Mit sämtlichen avirulenten Varianten erzeugten wir Kaninchenimmunserum von hohem Titer, das mit sämtlichen Varianten des Stammes vollkommen erschöpft werden kann. Letzten Endes gehen sämtliche Eigenschaften des Originalstammes — mit Ausnahme der Virulenz — unverändert in die morphologischen Varianten über. Das Erscheinen dieser Varianten lenkt somit die Aufmerksamkeit auf die Virulenzveränderungen des Stammes, und mit ihrer Isolierung vermag man auf einfache Weise Varianten mit veränderter Virulenz zu züchten.

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VERGLEICHENDE UNTERSUCHUNG VON MYCOBAKTERIEN TIERISCHER
UND MENSCHLICHER HERKUNFT
UND VON TUBERKELBAZILLUS-VARIANTEN

1. Wie die Untersuchungen ergaben, stammt ein Teil der vom Menschen gezüchteten atypischen Mycobakterienstämme, und zwar sowohl der photochromogenen wie der skotochromogenen Stämme, aller Wahrscheinlichkeit nach vom Tuberkelbazillus.

2. Aus Untersuchungen an Affen ging hervor, daß auch diese Tiere anscheinend Träger der atypische Tuberkulose verursachenden Mycobakterien sein können. Von den isolierten Stämmen wies der *Zol*-Stamm sowohl bezüglich des Amidase-Abbaus als auch hinsichtlich der Antigenstruktur und immunogenen Eigenschaften Unterschiede gegenüber den humanen und den Avian-Tuberkulosestämmen auf.

3. Die vom Tuberkelbazillus stammenden, durch Dissoziation entstandenen Stämme unterscheiden sich nicht nur in ihren Antigeneigenschaften, sondern auch in ihrem Amidasebestand und in ihrer Chemoresistenz wesentlich vom ursprünglichen Tuberkelbazillus; ihre Antigenstruktur zeigt jedoch auf ihren Ursprung deutende Merkmale.

4. Die vergleichende Untersuchung der von Mensch und Tieren gezüchteten Mycobakterien und der aus dem Tuberkelbazillus entstandenen Varianten liefert sehr wichtige Hinweise auf den Ursprung der atypischen Mycobakterien.

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ISOLIERUNG UND CHARAKTERISIERUNG EINIGER PHAGEN
DER *B. CEREUS*-SPEZIES

Wir isolierten bisher unbekannte Phagen der *B. cereus*-Spezies, so daß uns etwa 20 verschiedene Cereus-Phagen zur Verfügung stehen. Im Rahmen der Versuche stellten wir die Bedingungen für ihre Isolierung und Vermehrung fest. Die Charakterisierung der Phagen erfolgte durch Beschreibung der Plaque-Morphologie, Analyse der Antigeneigenheiten, durch Feststellung der qualitativen und quantitativen »Hostrange«-Verhältnisse und durch das Studium der lysogenen Eigenschaften. Mit Hilfe der Phagen reichten wir die *B. cereus*-Stämme in Gruppen ein, ferner suchten und fanden wir Zusammenhänge zwischen der Phagempfindlichkeit und chemischen Struktur der Zellwand.

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ANTIGENSTRUKTURELLE UND PHAGTYPISIERUNGSUNTERSUCHUNGEN
AN DEN STÄMMEN *E. COLI* O : 124 UND K : 72 (B : 17)

In den vorangegangenen Jahren untersuchten wir das Vorkommen der Stämme bei sporadischen Enteritiden und in Reihenuntersuchungen sowie die Ausscheidung der Erreger bei rekonvaleszenten und gesunden Ausscheidern.

Nach verschiedenen Phagtypisierungsmethoden führten wir Versuche zur Klassifizierung dieser Stämme durch.

Von den in der Literatur beschriebenen *E. coli*-Stämmen O : 124 und K : 72 (B : 17) mit verschiedenem H-Antigen sind die Erkrankungen anlässlich der Wasserepidemie in Szentgotthárd von einer geißelfreien Variante, bei den Wasserepidemien in Veszprém und Parádsasvár von Stämmen mit der Antigenstruktur H : 32 hervorgerufen worden.

Mit den in unserem Laboratorium gezüchteten mehreren hundert und den von anderen Laboratorien übergebenen Stämmen haben wir die H-Antigenbestimmung ausgeführt, um einen Überblick über die Verteilung sämtlicher Stämme, die anlässlich sporadischer Enteritiden und Reihenuntersuchungen in dem von unserem Laboratorium versorgten Bezirk gezüchtet wurden, nach ihrem H-Antigen zu gewinnen und mit den Angaben der von anderen Gebieten herrührenden Stämme vergleichen zu können.

Die Untersuchung und Klassifizierung unserer Stämme haben wir auch auf Grund ihrer latenten Phagen vorgenommen.

Mit den H-Antigen-Bestimmungen und Phagtypisierungsuntersuchungen wünschten wir auf das Vorkommen verschiedenartiger *E. coli* O : 124- und K : 72 (B : 17)-Stämme und auf die Möglichkeit der epidemiologischen Verwertung der Untersuchungsergebnisse hinzuweisen.

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PHAGUNTERSUCHUNGEN BEI KLEBSIELLA-ENTERITIDEN AUF EINER FRÜHGEBORENENABTEILUNG

1. Zwecks Klärung des Krankheitserregers der vom September 1961 bis zum April 1962 im Schöpf-Merei-Krankenhaus epidemisch aufgetretenen schweren Enteritiden wurden bakteriologische und Phaguntersuchungen durchgeführt. Aus dem Stuhl von 155 Frühgeborenen konnten Stämme der Klebsiella-Gruppe isoliert werden. Außer den morphologischen, biochemischen und Mäusepathogenitätsuntersuchungen wurde die Phagisolierung in 174 Fällen — bei 79 Personen — versucht. Bei den Paralleluntersuchungen vermochten wir neben 117 Klebsiella-Stämmen in 20 Fällen spezifische Klebsiella-Phagen nachzuweisen. Die Phagisolierungs- und Phagempfindlichkeitsuntersuchungen erwiesen sich als gut anwendbare Methoden zur Klarstellung bzw. zum Beweise der pathogenen Wirkung der fakultativen Krankheitserreger.

2. Bei der Untersuchung der im Verlauf der Epidemie isolierten 204 zur Klebsiella-Gruppe gehörenden Stämme mit 9 verschiedenen, von uns isolierten Phagen haben sich etwa 70% der Stämme als phagempfindlich erwiesen. Während der Epidemie verbreiteten sich Stämme von unterschiedlicher Phagempfindlichkeit. Beim Vergleich der Phagempfindlichkeit der von symptomfreien Ausscheidern und der von klinische Veränderungen aufweisenden Frühgeborenen herrührenden Stämme stellten wir fest, daß in den mit Symptomen einhergehenden Fällen in einem signifikant höheren Prozentsatz phagempfindliche Stämme vorkamen als in den symptomfreien Fällen.

Aus den bisherigen Ergebnissen darf auf einen Zusammenhang zwischen der Phagempfindlichkeit bzw. dem lysogenen Zustand der Klebsiella-Stämme und ihrer Pathogenität geschlossen werden.

VIROLOGIE**I. BÉLÁDI, M. BAKAY***(Mikrobiologisches Institut der Medizinischen Universität Szeged)***UNTERSUCHUNG DER PERSISTENTEN
AUJESZKY-VIRUS-INFEKTION IN KALBSNIERENZELLEN**

Kalbsnierenzellen, die mit dem im Hühnerembryogewebe gezüchteten Aujeszky-Virus infiziert werden, sind unter Bewahrung ihrer intakten Morphologie wochenlang zur Viruserzeugung imstande. Die Wertbestimmung des Virus geschieht auf Einzellschicht-Hühnerembryokulturen nach der Plaque-Methode. Kaninchenhirn-Virus ist nicht imstande, eine persistente Infektion herbeizuführen, indessen wird es, einmal durch Hühnerembryogewebe passierend, hierzu geeignet. Bei der Passage der persistent infizierten Kulturen mit Hilfe von Trypsin führen die Zellen das Virus mit sich, so daß Carrier-Kulturen hergestellt werden können. Zur Herbeiführung der persistenten Virusinfektion bedarf es auch der Anwendung von Kalbsserum in der Nährflüssigkeit. Die Kalbsnierenzellen, die mit dem im Hühnerembryogewebe gezüchteten Virus infiziert worden sind, werden in Anwesenheit von Kaninchenserum nicht persistent infiziert, sondern gehen, während sich zugleich die für die Viruswirkung charakteristischen Zellveränderungen allmählich entwickeln, in 4—5 Tagen zugrunde. In den mit Hühnervirus infizierten Kalbsnierenkulturen konnte am 3. Tage nach der Infektion Interferonbildung nachgewiesen werden, während die Interferonproduktion der persistent infizierten Zellen oder der Zellen der Carrier-Kulturen nicht festzustellen war.

GY. HADHÁZY, L. VÁCZI, É. HORVÁTH*(Mikrobiologisches Institut der Medizinischen Universität Debrecen)***UNTERSUCHUNGEN ÜBER DIE ZUSAMMENHÄNGE ZWISCHEN
DER VERMEHRUNG DES INFLUENZA-VIRUS
UND DER INTERFERON-BILDUNG**

Verff. untersuchten die Wirkung der Temperatur auf die Vermehrung des Influenza-Virus und auf die Interferon-Erzeugung. Nach Beimpfung eines 12 Tage alten Hühnerembryos mit dem Influenza-Virus-Stamm PR-8 in die Allantoishöhle wurde zu verschiedenen Zeitpunkten (in der 12., 24., 48., 72. und 96. Stunde) der Virusgehalt (infektiver Titer und Ha-Titer) und der Interferongehalt in der Allantoisflüssigkeit bestimmt.

Wie die Untersuchungen ergaben, wird die Virusvermehrung im Verhältnis zu den bei 35° C gezüchteten Eiern bei der Temperatur von 39—40—

41—42° C in zunehmendem Maße schwächer. Totale Vermehrungshemmung war selbst bei der Temperatur von 42° C nicht zu beobachten. Den Höhepunkt erreicht der Virusinfektionstiter im allgemeinen nach 24 Stunden, der Hämagglutinationstiter nach 48 Stunden. Bei höheren Temperaturen sinkt der Virusinfektionstiter im Verhältnis zur Temperaturerhöhung stürmisch. Mit der Temperaturerhöhung steigert sich die Interferonerzeugung bis zu einer gewissen Grenze. Von der höheren Temperatur wird der Vermehrungszyklus des Virus reversibel und in Richtung der Interferonproduktion verschoben, und diesem Mechanismus dürfte bei der auf die Influenza-Virusinfektion folgenden Genesung eine wesentliche Rolle zufallen.

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DIE WIRKUNG DES MODIFIZIERTEN FRANCIS-INHIBITORS
AUF DIE VERMEHRUNG DES INFLUENZA-VIRUS

Das aktive Influenza-Virus bindet sich an den aus Humanserum nach der HIRSTschen Methode hergestellten FRANCIS-Inhibitor und wird dann bei Zimmertemperatur im Verlauf der 6 Stunden des Versuches wieder frei. Die Virusbindung mit dem an die Anilinderivate gekoppelten Inhibitor erfolgt wesentlich konstanter, während der 6 Versuchsstunden bleibt sie bestehen. Deshalb hemmen diese Substanzen die Vermehrung des Influenza-Virus bei den in der Gewebeskultur und im Bruthennenei vorgenommenen Untersuchungen. In vitro haben sich die modifizierten Inhibitoren nicht als viruzid erwiesen.

Im Rahmen der bisherigen Versuche war der an Diäthyl-p-phenylendiamin gebundene FRANCIS-Inhibitor am wirksamsten, von dem wir auch die in Azeton lösliche Fraktion untersuchten. Der Hämagglutinations-Hemmungstiter des im Indikatorzustand untersuchten Lee-Virus betrug $\frac{1}{6}$ der Ausgangssubstanz, und seine in der Gewebeskultur bestimmte 75%ige Vermehrungshemmungskonzentration ergab denselben Wert. In vitro erwies sich diese Fraktion (8 ac) als viruzid und setzte den infektiösen Titer des im Verlauf der 6 Versuchsstunden über einen beträchtlichen Hämagglutinations-Resttiter verfügenden Influenza-Virus um etwa 5 $\log_{10}$ herab.

Den Hämagglutinationstiter der Nährflüssigkeit des deembryonierten Eies bestimmten wir nach der Mikromethode von TAKÁTSY. Wurde die Substanz 8 ac in der 5. Stunde der Latenzzeit dem System zugegeben, so führte sie noch immer eine bedeutende Vermehrungshemmung herbei. Wurde 8 ac nach 24 Stunden entfernt, so zeigte die Hämagglutininproduktion beträchtliche Regeneration, wenn auch der Kontrollwert nicht erreicht wurde. Durch entsprechende Wahl der quantitativen Verhältnisse konnte mit dem in der

1.—2. Stunde dem System zugesetzten 8 ac eine derartige Vermehrungshemmung erzielt werden, daß auch durch Entfernung der Hemmsubstanz nach 24 Stunden keine Regeneration der Hämagglutininproduktion zustande kam.

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UNTERSUCHUNGEN ÜBER DIE INTRAZELLULÄRE VERMEHRUNG DES VARICELLA-VIRUS

(Zusammenfassung nicht eingegangen)

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DAS STUDIUM DES VIRUSBEFALLS IN ZELLKULTUREN

1. Es wurde versucht, mit dem aus überimpfbarer Affenniere und He-La-Zellkultur gewonnenen Poliovirus 1. Typ LSc 2ab, 1. Typ Mahoney, 2. Typ MEF-1 und Coxsackie B-3 virustragende Zelllinien zu gestalten. In beiden Zellkulturen gelang es, Mahoney, LSc 2ab und MEF-1-Virus tragende Zelllinien zu bilden. Bei den Mahoney und MEF-1-Virus tragenden Zelllinien verwendeten wir homologes Immunserum, während dies bei der LSc 2ab-Virus tragenden nicht erforderlich war.

Von den gebildeten Zelllinien zeigte die das LSc 2 ab-Virus tragende Affennieren-Zelllinie Resistenz gegenüber $10^{6.50}$ TCD₅₀ des Poliovirus 1. Typ, $10^{-4.50}$ TCD₅₀ des 2. Typ, $10^{3.00}$ TCD₅₀ des 3. Typ und $10^{3.50}$ TCD₅₀ des Coxsackie B-3-Virus. Diese Resistenz beruhte auf Interferenz, bei deren Entwicklung die getragenen Viruspartikelchen und das Interferon gemeinsam eine Rolle spielten.

2. Aus der das Virus LSc 2ab tragenden Affennierenzelllinie konnte das Virus 3 und 6 Monate nach Beimpfung zurückgewonnen werden. Bei dem reisolierten und dem ursprünglichen LSc-Virusstamm untersuchten wir den genetischen Charakter der Vermehrung bei 40° C (*rct*/40), bei niedrigerer NaHCO₃-Konzentration (*d*), in überimpfbarer Affennierenzellkultur und in He-La-Zellkultur.

Es konnte folgendes festgestellt werden: a) Der *rct*/40- und *d*-Charakter der reisolierten Virusstämme hat sich nicht verändert.

b) Die reisolierten Stämme übten auf die überimpfbare Affenniere eine kräftigere zytopathogene Wirkung aus als der Original-Virusstamm LSc 2ab.

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ÜBER DEN MECHANISMUS DER »LOKAL ERWORBENEN RESISTENZ«
DER MIT VIRUS INFIZIERTEN PFLANZENBLÄTTER

Bekanntlich sind die Blattgewebe, welche die unter Wirkung einer Virusinfektion entstandenen lokalen Läsionen umgeben, der Superinfektion gegenüber resistent (erworbene lokale Resistenz). Die Atmung der resistenten Gewebe wird im Vergleich zu der der gesunden Gewebe desselben Blattes wesentlich stärker. Unsere Untersuchungen verfolgten den Zweck, durch eingehendes Studium der Atmung von Geweben, welche diese lokal erworbene Resistenz aufweisen, eine Erklärung für den Mechanismus der erworbenen lokalen Resistenz zu finden.

Wie festgestellt wurde, kommt es in den die Läsionen umgebenden Geweben zur starken Aktivierung folgender Enzyme: Glukose-6-P-dehydrogenase, 6-P-Glukonat-dehydrogenase, Zytochromoxydase, TPNH- und DPNH-Chinonreduktase und des TPNH-Oxydasesystems. In der Aktivität der Phosphohexoisomerase, Pentosephosphatisomerase und Apfelsäure-dehydrogenase haben wir keinen Unterschied feststellen können.

Aus diesen Ergebnissen darf geschlossen werden, daß hauptsächlich die Hexosemonophosphat-Shunt-dehydrogenasen aktiviert werden sowie diejenigen Systeme, welche die Elektronen von den reduzierten Dinukleotid-Koenzymen über die Polyphenole und die Polyphenoloxydase zum Sauerstoff der Luft liefern.

Die Aktivitätssteigerung obiger Enzyme in den die nekrotischen Flecke umgebenden Geweben ist wahrscheinlich eine Folgeerscheinung der Proteinsynthese. Bestätigt wird diese Vermutung durch unsere mit P^{32} durchgeführten Untersuchungen, da in den fraglichen Geweben gesteigerte Ribonukleinsäure-Synthese festgestellt werden konnte.

Die Resultate gestatten folgende Schlußfolgerungen:

1. Die erworbene lokale Resistenz dürfte auf der Kompetition der Virus- und Enzymproteinsynthese beruhen.
2. Eine Rolle spielt möglicherweise die Hemmung der zur Entwicklung der lokalen Läsionen erforderlichen Chinonanhäufung durch die Aktivierung der Enzymsysteme (TPNH-Chinonreduktase, DPNH-Chinonreduktase), die an der terminalen Oxydation teilnehmen.

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UNTERSUCHUNG DER WIRKSAMKEIT VON POCKENIMPFSTOFF
DURCH INTRAKUTAN- UND KUTANIMPFUNG VON KANINCHEN
SOWIE DURCH BEIMPFUNG DER CHORIOALLANTOIS-MEMBRAN
VON HÜHNEREMBRYOS

Es wird über Erfahrungen berichtet, die bei der Bestimmung der Wirksamkeit von Pocken-Vakzine mit der Intrakutan- und Kutanimpfung von Kaninchen sowie mit der Inokulation der Chorioallantois-Membran von ausgebrüteten Hühnereiern gemacht wurden, weiterhin über Beobachtungen, die sich im Verlauf der Anwendung dieser drei Methoden im Zusammenhang mit der Gewebspathogenität des zur Impfstoffherstellung dienenden Vaccinia-Virusstammes ergaben.

Nach intrakutaner Zufuhr der Pocken-Vakzine-Verdünnungen erreicht die vakzinöse Veränderung am 4.—5. Tage den Höhepunkt, doch kommt es vor, daß das Maximum bei stärkeren Verdünnungen am 6.—7. Tage in Erscheinung tritt, wodurch die Auswertung erschwert wird. Unter den einzelnen Kaninchen treten große individuelle Unterschiede zutage. Neben dem untersuchten Impfstoff wurde auch eine Reference-Vakzine (lyophilisierte Eier-Vakzine) benutzt. Auf diese Weise wurde ein brauchbareres, aber auch nicht immer reproduzierbares Resultat gewonnen. Die Methode orientiert gut über die Gewebspathogenität des Virus. Die Hautinfiltration ist umschrieben, die Rötung schwach, bei konzentrierterer Anwendung stärker. Eine ausgedehntere Nekrose wurde niemals beobachtet, als Zeichen dafür, daß das Virus nach den vielen Kalb-Kaninchen-Kalb-Passagen keine gesteigerte Gewebspathogenität angenommen hat.

Bei Anwendung der Reference-Vakzine ergab die Kutanmethode besser bewertbare Ergebnisse als das Intrakutanverfahren, doch konnten die Resultate nicht immer reproduziert werden. Das Zentrum der Pusteln begann am 5—6. Tage zu nekrotisieren, der Schorf fiel am 12.—14. Tage ab und hinterließ eine oberflächliche, glänzende Narbe. Für Vaccinia-Stämme mit erhöhter Gewebspathogenität charakteristische, tiefe Nekrose aufweisende Pusteln, die auch nach 3—4 Wochen post infectionem noch fest an der Haut haften und nach ihrer Abstoßung eine tiefe Narbe zurücklassen, wurden nicht beobachtet.

Die Zahl der Herdbildungseinheiten wurde durch Beimpfung von 11—12 Tage alten Hühnerembryos nach der Methode von WESTWOOD bestimmt. Die Ablesung erfolgte nach 42—48 Stunden. Die Herdzahl der im Verkehr befindlichen, d. h. staatlich kontrollierten Pockenimpfstoffe, die sich bei den zum ersten Mal geimpften Personen als geeignet erwiesen haben, wurde mit $3,5 \times 10^6$ — $3,2 \times 10^7$, im Mittelwert mit $1,1 \times 10^7$ festgestellt. Auf gesteigerte Gewebspathogenität deutende hämorrhagische Veränderungen wurden an den Chorioallantois-Membranen niemals wahrgenommen.

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VIRUSISOLIERUNGSVERSUCHE ZUR ÄTIOLOGISCHEN KLÄRUNG DER
EINHEIMISCHEN KERATOCONJUNCTIVITIS-EPIDEMIE

Im Verlauf der Untersuchung des Conjunctiva-Abstrichs von 25 an Conjunctivitis epidemica erkrankten Personen in der Hela-Zellkultur isolierten wir ein zytopathogenes Agens, das neben der charakteristischen Einschlußkörperchenbildung durch die langsame Vermehrung und die in Herden auftretende Gewebsdegeneration gekennzeichnet ist.

Die mit den Eigenschaften dieses Virus zusammenhängenden Beobachtungen werden mitgeteilt.

Nach den bisherigen Untersuchungen wird das Virus von den zur Verfügung stehenden 13 Adenovirustypus-Sera nicht neutralisiert. Die serologischen Untersuchungen zwecks Klarstellung der ätiologischen Rolle des Virus sowie die Untersuchungen zur genauen Typusbestimmung sind gegenwärtig im Gange.

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VIRUSISOLIERUNGSVERSUCHE
BEI PHARYNGOKONJUNKTIVALEM FIEBER

Im August 1961 wurden bei Kindern gehäuft vorkommende Pharyngoconjunctivitis-Erkrankungen beobachtet. In 13 Fällen versuchten wir, die Ätiologie zu klären. Aus 16 Materialien von 9 Personen gelang die Virusisolierung in der He-La-Gewebskultur. Von 13 Augensekreten erwiesen sich 7, von 18 Rachensekreten 5, von 8 Stuhlproben 4 als positiv. Die gezüchteten Virusstämme gehörten mit einer Ausnahme zur Adenovirus-3-Gruppe.

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ISOLIERUNG DES PARAINFLUENZA-VIRUS
AUS RESPIRATORISCHEN INFEKTIONEN IM SÄUGLINGESALTER

Im Verlauf des Studiums der Respirationserkrankungen im Säuglings- und Kindesalter isolierten wir unter Anwendung permanenter Affennierenzellkultur und mit Hilfe der Hämaadsorptionstechnik Myxovirus aus der Rachen-

waschflüssigkeit von 2 Säuglingen. Mit den in Hühnern erzeugten Immunsera der Viren Influenza- A_1 (PR-8), A_2 (Singapore), B (Lee) und C (Taylor) fiel der Ha-Hemmungstest der Viren negativ aus. Die Parainfluenza 2- und 3-Kaninchenimmunsera ergaben gleichfalls einen negativen Ha-Hemmungstest mit den neu isolierten Viren, und von den mit letzteren produzierten Kaninchen-Immunsera wurde die Hämagglutination des Parainfluenza-Virus 2 und 3 ebenfalls nicht gehemmt. Das Parainfluenza 1-Kaninchenimmunserum und die mit den beiden neu isolierten Viren erzeugten Immunsera ergaben sowohl mit dem Parainfluenza-Virus 1 als auch mit den neu isolierten Viren einen Ha-Hemmungstest mit positivem Resultat. Von den einzelnen Sera wurde die Hämagglutination der erwähnten Viren in gleichem Maße gehemmt. Diese Ergebnisse konnten in Neutralisationsversuchen in der permanenten Affen-nierenzellkultur bestärkt werden. Auf Grund des mit Parainfluenza-Virus 1 übereinstimmenden serologischen Verhaltens im Verlauf der durchgeführten Untersuchungen halten wir beide Viren für Parainfluenza-Virus 1.

Einer der viruspositiven Säuglinge litt an Katarrh der oberen Luftwege, der andere an Bronchopneumonie. Pathogene Bakterien konnten in ihrem Rachensekret nicht nachgewiesen werden. Wir hatten Gelegenheit, das paarige Blutserum des an Bronchopneumonie erkrankten Säuglings serologisch zu untersuchen. Im zweiten Blutserum konnte ein signifikant erhöhter Ha-Hemmungs- und Komplementbindungs-Antikörpertiter dem Parainfluenza-Virus 1 gegenüber im Verhältnis zur ersten Blutprobe festgestellt werden, während den Parainfluenza 2-, 3-, Mumps-, Influenza-A-, B- und C-Adenoviren gegenüber Antikörpervermehrung (mit der Ha-Hemmungs- bzw. Komplementbindungsprobe) nicht aufzuzeigen war. Die ätiologische Rolle des Parainfluenza-Virus 1 bei den fraglichen beiden Erkrankungen muß auf Grund der durchgeführten Untersuchungen als wahrscheinlich bezeichnet werden.

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HERSTELLUNG EINES LYOPHILISIERBAREN IMPFSTOFFS GEGEN FLECKTYPHUS

Nach den bisherigen Erfahrungen erleidet der gegenwärtig benutzte Flecktyphus-Impfstoff bei der Lyophilisierung eine beträchtliche Wertverminderung und kann seine Wirksamkeit auch ganz verlieren, was wahrscheinlich auf der schwachen eiweißdenaturierenden Eigenschaft des im Impfstoff enthaltenen Phenols beruht. Anscheinend wird durch die Anwesenheit von Phenol über einer gewissen Konzentration die Wiederauflösung der Eiweißstoffe gehemmt, ungeachtet dessen, daß bei der Lyophilisierung ein großer Teil des

Phenolgehalts im Impfstoff verdunstet. Die Herstellung eines lyophilisierbaren Impfstoffs schien daher wünschenswert. Im Verlauf unserer Arbeit stellten wir verschiedene Impfstoffproben mit niedrigerem Phenolgehalt als üblicherweise her. Als die mit der Komplementbindungsprobe bestimmten Antigentiter dieser Impfstoffproben mit den Antigentitern derselben Proben nach der Lyophilisierung verglichen wurden, ergab sich, daß der Antigentiter bei 0,20%iger oder noch niedrigerer Phenolkonzentration unverändert geblieben war. Nunmehr wurde ein Versuchsimpfstoff mit 0,13%igem Phenolgehalt hergestellt und lyophilisiert, der den Anforderungen entspricht, die an die Wirksamkeit eines Flecktyphus-Impfstoffs gestellt werden müssen. Die Anwendbarkeit des Flecktyphus-Impfstoffs, die bisher 12—15 Monate betrug, dürfte somit wesentlich verlängert werden.

MYKOLOGISCHE, INDUSTRIELLE UND LANDWIRTSCHAFTLICHE MIKROBIOLOGIE

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ÜBER AKTUELLE FRAGEN DER ALGEN-MASSENZÜCHTUNG

(Referat. Zusammenfassung nicht eingegangen)

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DIE WIRKUNG IONISIERENDER STRAHLEN AUF DIE MYCELIEN DER PEKTOLYTISCHE ENZYME ERZEUGENDEN ASPERGILLUS-STÄMME

Untersucht wurde die Wirkung der Bestrahlung von *Aspergillus foetidus*-Stämmen auf die Permeabilität der Mycelien. Von größeren Strahlendosen wurde die Aminosäuren-Permeabilität der Zytoplasmamembran stark erhöht. Eine Steigerung der Enzymdiffusion war bei dieser Gelegenheit nicht zu beobachten.

Die im Infrarotspektrum der Mycelien und ihrer Extrakte unter Wirkung der Bestrahlung zustande gekommenen Veränderungen wurden gleichfalls ausgewertet.

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BIOCHEMISCHE UNTERSUCHUNG DER TAXONOMISCH BEWERTETEN
PHYSIOLOGISCHEN EIGENTÜMLICHKEITEN VON HEFEPILZEN

Vor einigen Jahren begannen wir die biochemischen Grundlagen der Kohlenhydratgärungs- und Kohlenhydratassimilationsproben, die bei der physiologischen Systematisierung der Hefepilze angewendet werden, zu untersuchen. Einer Lösung der Frage suchten wir einerseits durch theoretische Konklusionen, andererseits in experimentellen Untersuchungen näherzukommen. Über mehrere Teilergebnisse sowohl der theoretischen Ableitungen wie der experimentellen Untersuchungen haben wir bereits an verschiedenen Stellen berichtet. Hier wollen wir an Hand der neuesten Versuchsergebnisse ein zusammenfassendes Bild über unsere bisherigen Resultate geben.

Die Untersuchungen wurden mit den lebenden, azetonbehandelten und homogenisierten Zellen der folgenden 6 Arten durchgeführt: *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Candida melibiosi*, *Candida pseudotropicalis* und *Candida solani*. Diese Arten repräsentieren teils völlig abweichende, teils verwandte Stoffwechseltypen und sichern daher einen ziemlich breiten, aber zusammenhängenden Überblick über die Frage.

Nach den verschiedenen Saccharosen gruppiert, vermochten wir folgenden festzustellen:

1a. Die Raffinosegärung findet zu $\frac{1}{3}$ oder zu $\frac{3}{3}$ statt, eine $\frac{2}{3}$ -Gärung haben wir nicht beobachtet (andere — seit mehreren Jahren vor sich gehende — Untersuchungen bestätigen diese Feststellung).

1b. Wenn die Raffinosegärung zu $\frac{3}{3}$, d. h. total stattfindet, so geht die Vergärung periodisch, in 3 Stufen vor sich. Dieser Prozeß kann bei einzelnen Arten prolongiert verlaufen; von diesen hatten wir früher mit Hilfe von ungenauen Methoden festgestellt, daß sie die Raffinose zu $\frac{2}{3}$ vergären.

1c. Bei der Spaltung der Raffinose zu Monosacchariden handelt es sich bis zuletzt um einen extrazellulären Prozeß, der von zwei hydrolysierenden Enzymen, der Invertase (β -h-Fruktosidase) und der Melibiase (α -Galaktosidase) durchgeführt wird.

1d. Die Raffinosegärung zu $\frac{3}{3}$ geht immer, die zu $\frac{1}{3}$ überwiegend mit Saccharosegärung einher. Im letzteren Fall findet dann keine Saccharosegärung statt, wenn der Raffinoseabbau ausschließlich von Melibiase ausgeführt wird, d. h. die Zellen keine Invertase produzieren.

2a. Die Saccharosegärung kann auf zwei Wegen in Gang kommen: durch extrazellulären Invertase-Abbau oder durch Permeation mit intrazellulärer Maltase-Spaltung (α -Glukosidase). Aus diesem Grunde geht die Saccharosegärung im allgemeinen mit Raffinose- oder Maltosegärung einher. Da jedoch

die Saccharose- und die Maltose-Permeation von besonderen Permeasen gefördert wird, kann bei der von α -Glukosidase herbeigeführten Spaltung auch der Fall vorkommen, daß die Maltosegärung nicht von Saccharosegärung begleitet wird (*Candida solani*).

2b. Die auch zum Saccharoseabbau fähige Maltase (α -Glukosidase) ist ein konstitutives, die Saccharosepermease hingegen ein adaptives Enzym. Indessen hängen entweder die Adaptationsverhältnisse stark von den anaeroben bzw. aeroben Bedingungen oder von besonderen anaeroben und aeroben Verhältnissen ab, oder es funktionieren besondere anaerobe und aerobe Permease-Enzyme.

3a. Die Maltosegärung setzt mit Permeation und mit nachfolgender intrazellulärer Maltase- (α -Glukosidase-) Hydrolyse ein.

3b. Die Maltase kann auch in Zellen nachgewiesen werden, die Maltose nicht vergären; das in Punkt 2b über die Permeation der Saccharose Gesagte gilt also auch hier.

4a. Die Laktosegärung beginnt ebenso wie die Maltosegärung (Punkt 3b) mit Permeation, der intrazelluläre Laktase- (β -Galaktosidase-) Hydrolyse nachfolgt.

4b. Die Permeation der Laktose ist gleichfalls ein aktiver Prozeß, der in Abhängigkeit von den Aervationsverhältnissen steht.

Darüber hinaus haben wir in mehreren Fällen beobachtet, daß unter der Wirkung einzelner spaltender Enzyme Oligosaccharide synthetisiert werden, so daß diese Enzyme also auch als Transglukosidasen funktionieren.

Die Ergebnisse, welche die Grundlagen vorstehender Schlußfolgerungen bilden, werden mit Chromatogrammen demonstriert, die anlässlich der verschiedenen Untersuchungen hergestellt worden sind.

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DIE AUSWAHL VON SCHIMMELPILZEN MIT HOHER GLUKOSEOXYDASE-AKTIVITÄT UND DIE FESTSTELLUNG IHRER OPTIMALEN ZÜCHTUNGSBEDINGUNGEN

Die Säureproduktion von etwa 80 *Aspergillus*- und *Penicillium*-Stämmen verschiedener Herkunft wurde untersucht. Der die meiste Glukonsäure erzeugende Stamm wurde auf Nährböden mit verschiedenem Glukose- und CaCO_3 -Gehalt in Oberflächenkulturen gezüchtet. Nach den Untersuchungsergebnissen über die Enzymaktivität der Mycelien macht die Aktivität der in der Nährlösung mit 5% Glukosegehalt in Anwesenheit von 6% CaCO_3 gezüchteten Mycelien etwa das Fünffache der ohne CaCO_3 gezüchteten aus. Bei Submers-

züchtung wurde die pH-Senkung durch periodische Zugabe von NaOH verhindert. Auf diese Weise konnten im Vergleich zu der CaCO_3 -freien Oberflächenkultur Mycelien mit 7fach stärkerer Aktivität gewonnen werden (500 E/g Trockensubstanz). Während bei der Oberflächenzüchtung nur etwa 70% sämtlicher produzierten Enzyme im Mycel anzutreffen sind, beläuft sich diese Menge bei der Submerskultur auf etwa 90%. Bei der Erschließung der Mycelzellen läßt sich das erzeugte Enzym größtenteils herauslösen und durch Fällung mit Isopropylalkohol ein hochaktives pulverförmiges Glukoseoxydase-Enzym (1500—4000 E/g) gewinnen.

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DIE ZUSAMMENHÄNGE ZWISCHEN DEN IM BODEN LEBENDEN
CTENOMYCES TRICHOPHYTICUS UND *MICROSPORON GYPSEUM*
UND DEN PFLANZENASSOZIATIONEN
AUF DER BODENOBERFLÄCHE SOWIE DER BODENMIKROFLORA

Auf Grund einer tabellarischen Zusammenstellung wurden gewisse Korrelationen zwischen den Bodentypen, ihrer Pflanzenassoziation an der Oberfläche und einzelnen Mitgliedern der Bodenmikroflora gefunden.

I. Im Sandboden kommen sowohl *Ctenomyces trichophyticus* wie *Microsporon gypseum* häufig vor. *Ctenomyces* befinden sich aber *Microsporon* gegenüber im Übergewicht. Der Sandboden enthält wenige Bakterien, jedoch reichlich *Streptomyces*. Die Verhältniszahl Bakterien/Pilze ist sehr niedrig. Ihre Verringerung begünstigt die *Ctenomyces*, ihre Vergrößerung hingegen nicht. Die Bodenoberflächenvegetation besteht nur aus einigen Grasgewächsen (hauptsächlich *Poa*-, *Fumana*-, *Festuca*-Arten).

Die führenden Pflanzenarten auf den Bleichsandböden sind die *Poa*- und *Festuca*-Arten.

II. Auf den zugrunde gehenden Luzernfeldern vermögen sich einzelne Grasarten anzusiedeln. In diesen Böden leben viele Insekten und Nagetiere, auch kommen ziemlich reichlich *Ctenomyces trichophyticus* in ihnen vor. Gehemmt wird ihre Anwesenheit durch die höhere Bakterien/Pilze-Verhältniszahl.

Im Boden der brachliegenden Wiesen sind in der Regel *C. trichophyticus* häufig anzutreffen.

Infolge des reichen Bakteriengehalts der Ackerböden wird *C. trichophyticus* zahlenmäßig in den Hintergrund gedrängt, *Microsporon gypseum* aber nicht.

III. Die Oberflächenvegetation der *Solonchak* besteht aus einer der *Festuca* und *Camphorosum*-, ev. *Puccinella*-Arten. Der Bakteriengehalt ist niedrig

(150 000—350 000), *Streptomyces* bilden 80—100% der Mikroflora. Die Solonetzböden haben einen ansehnlichen Bakteriengehalt. Sie weisen weniger *Streptomyces* auf als die »*Solonchak*«, aber noch weniger *C. trichophyticus*. Die führenden Pflanzen ihrer Oberflächenvegetation sind einzelne Mitglieder der *Festuca*-, *Statice*- und *Inula*-Arten, weiterhin *Camphorosma annua*, *Artemisia maritima* und noch einige Alkalibodenpflanzen.

IV. Waldböden.

1. Die Oberflächenvegetation des Sandbodens der Robinien (Akazien) besteht aus Pflanzen der *Poa*- und *Festuca*-Arten, hauptsächlich aus *Poa pratensis*, *Poa angustifolia*, *Poa nemoralis*, *Festuca valesiaca*, *Festuca pratensis*. In diesen Böden kommt *C. trichophyticus* reichlich vor, *Microsporon gypseum* bleibt weit dahinter zurück. Im Hinblick auf den geringen Bakteriengehalt ist die Verhältniszahl Bakterien/Pilze sehr niedrig. *Actinomyces* (*Streptomyces*) machen 30—50% der Bodenflora aus. Die Häufigkeit des Vorkommens von *C. trichophyticus* in Sandböden ist absolut, nicht nur relativ.

In den sandig-alkalischen Waldböden stimmen die Verhältnisse im großen und ganzen mit denen der Robinienböden überein.

Im Boden der Bergweiden ist *C. trichophyticus* reichlich vorhanden. Auf diesen herrschen die Grasgewächse vor, vor allem *Festuca glauca*, *Festuca sulcata*, *Festuca valesiaca*, *Melica ciliata*, *Poa pratensis*, weiterhin *Koeleria glauca*, *Koeleria gracilis* und *Chrysopogon gryllus*.

Zusammenhänge zwischen den biologischen Merkmalen und der Mikroflora der verschiedenen Bodentypen:

I. In Sand-, Lehm- und Alkaliböden:

1. Die Pflanzenassoziationen an der Oberfläche sind sehr ärmlich und zeigen viele gemeinsame Züge.

2. Ihre Bakterien beschränken sich auf einige Arten, und im Vergleich zu denen der Humusböden ist ihre Gesamtzahl niedrig. Die Verringerung des Gegensatzes zwischen Bakterien und Pilzen zieht die Vermehrung der Pilzmengen nach sich, ihre Steigerung dagegen die Verminderung der Pilze.

3. Derartige Böden enthalten sehr häufig *C. trichophyticus*, im Vergleich dazu spärlicher *Microsporon gypseum*.

II. In den Humusböden manifestiert sich die Wirkung der Bakterienvielfalt stark *C. trichophyticus* gegenüber, jedoch in viel geringerem Maße *Microsporon gypseum* gegenüber, weshalb in diesen Böden wenige *C. trichophyticus*, hingegen viel mehr *Microsporon gypseum* anzutreffen sind. Niedrig ist auch das prozentuale Vorkommen von *Streptomyces*.

Unter Berücksichtigung der obigen Angaben können auf Grund der Qualität und Pflanzenassoziationen auf der Oberfläche des Bodens Schlussfolgerungen auf die Zusammensetzung der Mikroflora des Bodens, insbesondere auf die Häufigkeit der Anwesenheit von *C. trichophyticus* gezogen werden.

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ÖKOPHYSIOLOGISCHE UNTERSUCHUNG
UND SYSTEMATISCHE DETERMINATION DER STREPTOMYCES-FLORA
IN DER MULLARTIGEN WALDRENDZINA

In den letzten Jahren wurden in zahlreichen Arbeiten die »Artlisten« der Strahlenpilzflora verschiedener Böden angegeben. Die Bestimmung der Strahlenpilze stößt indessen auf Schwierigkeiten, und unter demselben Artnamen hat man die verschiedensten Organismen beschrieben, so daß es auf Grund eines Vergleichs der Literaturangaben kaum möglich ist, die tatsächlichen Differenzen zwischen den Bodenmikroflora zu beurteilen. Es wurde versucht festzustellen, inwieweit die Möglichkeit besteht, die Strahlenpilzflora eines Bodens systematisch zu bestimmen und seine ökophysiologischen Eigentümlichkeiten zu erkennen. Zu diesem Zweck wurden die folgenden, sich auf mehrere Jahre erstreckenden Arbeiten durchgeführt: 1. Aus zahlreichen isolierten Stammmaterialien wurden 34 Streptomyces-Arten selektiert. 2. Die Stammprepräsentanten dieser Arten wurden in bezug auf mehr als 300 physiologische und kulturelle Eigenschaften untersucht. 3. Die Isolate wurden direkt mit 400 internationalen authentischen Strahlenpilzstämmen einer Sammlung verglichen. 4. Zur Identifizierung der Stämme wurden ausgedehnte serologische Untersuchungen mit der von MÉSZÁROS für Strahlenpilze modifizierten Hämagglutinationsprobe von MIDDLEBROOK und DUBOS vorgenommen.

Nach den Ergebnissen besteht die Strahlenpilzflora des fraglichen mullartigen Rendzinabodens aus den an die spezielle Rendzina-Dynamik adaptierten Arten. Etwa 80% der isolierten Arten konnten auf Grund der wichtigsten kulturell-physiologischen Merkmale bestimmt werden, indessen war kein Stamm zu beobachten, der vom Gesichtspunkt des untersuchten »Eigenschaftsmosaiks« völlige Identität mit der sog. »Typus«-Kultur einer der bekannten Arten gezeigt hätte. Verantwortlich hierfür ist die Variabilität der Mikroorganismen und ihre Anpassung an die Umweltverhältnisse (Mutation, Selektion, Adaptation). Serologisch gelang es vor allem den näheren Verwandtschaftskreis einzelner Stämme klarzustellen, doch konnte auch außerordentlich enge Identität beobachtet werden.

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BIOCHEMISCHE UNTERSUCHUNG VON RHIZOBIUM-ARTEN (-GRUPPEN)

Mit Hilfe der Bakteriendeterminanten können gewisse Bakterienarten (vor allem die zur medizinischen Bakteriologie rechnenden sog. pathogenen Arten) identifiziert werden, während die Bestimmung anderer Arten (die im Bereich der landwirtschaftlichen und industriellen Bakteriologie eine wichtige Rolle spielen) nach den Bakteriendeterminanten heute noch — angesichts der mangelhaften und unsicheren Kenntnis ihrer Merkmale — in vielen Fällen unsicher, in anderen unmöglich ist.

Letzteres bezieht sich auch auf die Rhizobien, deren systematologische Position noch nicht akzeptiert werden kann. In den früheren und neueren Bakteriendefinitionen sind die Charakteristika zur Identifikation der Rhizobien sehr abweichend und mangelhaft angegeben. Um diese Lücken auszufüllen, wurden Versuche durchgeführt.

Die untersuchten 45 Rhizobium-Stämme konnten auf Grund ihres biochemischen Verhaltens in 3 Gruppen eingereiht werden. Die Stämme der Gruppe A sind dadurch gekennzeichnet, daß sie Glukose, Galaktose, Xylose, Lävulose, Saccharose, Raffinose und Salizyl überhaupt nicht angreifen. Bezeichnend für die Stämme der Gruppe B ist, daß sie aus den eben erwähnten Verbindungen Säure und Gas, aber aus Eskulin nur Säure erzeugen. Kennzeichnend für die Stämme der Gruppe C ist hingegen, daß sie im Gegensatz zu den B-Stämmen aus den erwähnten Verbindungen nur Säure produzieren, Eskulin aber nicht abbauen.

Die Rhizobium-Stämme konnten in eine dieser 3 Gruppen eingeordnet werden, unabhängig davon, an den Wurzeln welcher Pflanzenart die Knollen gefunden wurden, aus denen wir sie gezüchtet haben. Nach unseren Versuchsergebnissen besteht nicht die Möglichkeit, die Rhizobien nach ihrem pflanzlichen Ursprung in verschiedene Arten aufzuteilen.

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UNTERSUCHUNG DER WECHSELWIRKUNG VON KUNSTSTOFF-FOLIEN
UND MIKROBEN

(Zusammenfassung nicht eingegangen)

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SYSTEMATOLOGISCHE UNTERSUCHUNG DER AUS KONSERVENSCHINKEN
(HALBKONSERVEN) GEZÜCHTETEN KOKKENSTÄMME

Aus Präserven wurden mehrere, morphologisch kokkenartige Stämme gezüchtet, die in ihren biochemischen Eigenschaften weitgehend mit den Merkmalen von *Streptococcus faecalis* übereinstimmten, aber von einigen Ausnahmen abgesehen keine Präzipitation mit dem Lancefield D-Serum gaben.

Auf Grund der eingehenden Analyse ihrer biochemischen und morphologischen Eigenschaften konnten die Stämme als *Aerococcus viridans* (WILLIAMS u. Mitarb.) identifiziert werden. Im Interesse einer auf breiteren Grundlagen ruhenden Identifizierung nahmen wir auch immunologische Untersuchungen vor.

Mit den Stämmen wurde Kaninchen-Immunsrum hergestellt. Die Autoagglutination der Stämme störte die Agglutinationsprobe, weshalb wir die Präzipitation erprobten. Unser Immunsrum gab mit dem zur Produktion benutzten Stamm im Mikropräzipitationsröhrchen ein positives Resultat bei den Serumverdünnungen 0,1 : 2 und 1 : 4, während das Ergebnis mit den anderen Stämmen zweifelhaft ausfiel. Es wurde auch die indirekte Hämagglutinationsprobe ausgeführt, bei der die eigenen Stämme ebenso wie der WILLIAMSsche *Aerococcus viridans*-Originalstamm bis zur Verdünnung 1 : 8 positive Resultate ergaben.

Wir haben die Differentialdiagnostik des *Aerococcus viridans*, insbesondere zwecks sicherer Differenzierung von *Streptococcus faecalis*, mit dem das Bakterium am leichtesten verwechselt werden kann, ausgearbeitet. Dieser kommt im Interesse der Konservierung und hygienischen Qualifizierung des Schinkenproduktes große wissenschaftliche und praktische Bedeutung zu.

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DIE WIRKUNG IONISIERENDER STRAHLUNG
AUF DIE MIKROFLORA VON GEWÜRZPAPRIKAHAHLPRODUKTEN

Es wurde untersucht, welche Veränderungen im Verlauf der mit ionisierender Strahlung vorgenommenen Keimzahlverminderung im Gewürzpaprikamahlgut in der qualitativen Zusammensetzung der Mikroflora eintreten.

Bei der überwiegenden Mehrzahl der untersuchten Paprikaproben besteht die Mikroflora zumeist aus verhältnismäßig strahlenempfindlichen Mikro-

organismen, weshalb eine 99,0—99,9%ige Keimzahlenkung bereits mit einer Strahlendosis von 300—400 r herbeigeführt werden kann. Am besten werden die Strahlen von den subtilis-mesentericusartigen Bakterien der Mikroflora toleriert. Bei dem mit diesen Mikroben erheblich stärker als üblicherweise verunreinigten Mahlgut (etwa 10^6 kg) kommt es unter Wirkung von 400 r zu einer etwa 90%igen Keimzahlverminderung. Infolge der unterschiedlichen Strahlenresistenz der einzelnen Komponenten der Mikroflora verändert sich unter Wirkung der erhöhten Strahlendosen auch die qualitative Zusammensetzung der Residual-Mikroflora und verschiebt sich zugunsten der sporentragenden Bakterien.

Nach den Untersuchungsergebnissen wird die Strahlenempfindlichkeit der Mikroben vom relativen Feuchtigkeitsgehalt des Mahlgutes nicht beeinflußt.

IMMUNOLOGIE

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NEUE BEITRÄGE ZUM MECHANISMUS DES SHWARTZMAN-PHÄNOMENS

(Referat. Zusammenfassung nicht eingegangen)

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HYPOTHERMIE UND ANAPHYLAXIE

In Meerschweinchenversuchen vermochten wir nachzuweisen, daß der aktive und passive anaphylaktische Schock von tiefer Hypothermie (22—23° C) gehemmt wird. Unserer Meinung nach läßt die Tätigkeit des histaminfreisetzenden spezifischen Enzymsystems im hypothermischen Zustand beträchtlich nach. Die Unterkühlung beeinflußt nicht den Histaminspiegel im Blutplasma der Versuchstiere. Die Hypothermie hemmt den auf Histaminfreisetzung beruhenden Jancsó-Test und die von Adrenalin herbeigeführte Histaminfreisetzung. Der mit Pferdeserum bei Hunden zustande gebrachte anaphylaktische Schock wird durch die Unterkühlung hochgradig gehemmt, und in diesem Fall zeigt auch der Bluthistaminspiegel keine wesentliche Erhöhung. Durch die Unterkühlung wird nicht nur die Freisetzung von Histamin verhindert, sondern auch der Ablauf der Antigen-Antikörperreaktion verändert.

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NEUERE UNTERSUCHUNGSERGEBNISSE ÜBER DIE BEZIEHUNGEN
DER SERUMEIWEISS-STOFFE UND ANTIKÖRPER

Zwecks Studiums der Zusammenhänge zwischen der Antikörperwirkung und dem Eiweißtypus wurden Plasmaproben und Sera von Menschen und Tieren bzw. die aus diesen mit verschiedenen präparativen Methoden gewonnenen Fraktionen eingehend untersucht.

I. Das zur Fraktionierung menschlicher Plasmagemische ausgearbeitete Verfahren hatten Verff. auf dem Kongreß 1961 der Ungarischen Mikrobiologischen Gesellschaft mitgeteilt. In den auf diese Weise hergestellten Präparaten bestimmten sie die elektrophoretische Motilität der Plasmaeiweißstoffe in der Antweilerschen Apparatur und ihre Antigenität mit stationären Immundiffusions- und immunelektrophoretischen Methoden bei gleichzeitiger Feststellung des Eiweißgehalts. Der Titer von mehr als 20 verschiedenen Antikörpern wurde *in vivo* oder *in vitro* untersucht. Die Ergebnisse führten zu den folgenden wichtigeren Feststellungen:

a) In den einzelnen Plasmafraktionen können auf Grund der Verteilung und Konzentration der verschiedenen Antikörper mindestens 4 Kategorien gebildet werden. Eine Fraktion enthält hauptsächlich Eiweißstoffe, die gamma-Globulin-Antigenität aufweisen, aber über keine Antikörperwirkung verfügen.

b) An Hand der Angaben darf als wahrscheinlich angenommen werden, daß die auf Grund ihrer Antigenität gut differenzierbaren gamma-, β_{2A} - und β_{2M} -Globuline nicht nur hinsichtlich ihrer elektrophoretischen Motilität bzw. Chromatographierbarkeit, sondern auch bezüglich ihrer Fraktionierbarkeit heterogen sind und daher in weitere Untergruppen aufgeteilt werden können.

II. Das Serum von Pferden, die mit Diphtherie-Antigenen hyperimmunisiert wurden, wurde bei gleichzeitiger Bestimmung des Antitoxingehalts mit elektrophoretischen und immunelektrophoretischen Methoden untersucht, wobei sich die folgenden Ergebnisse ergaben:

a) Bei hyperimmunisierten Pferden ist die Antitoxinwirkung im Serum an das intermediäre Motilität aufweisende T-Globulin gebunden. Die Korrelation zwischen den beiden Eigenschaften ist signifikant.

b) Die unter Wirkung intensiver Hyperimmunisierung eintretende Vermehrung von T-Globulin führt zur kompensatorischen Senkung des Spiegels der anderen Serumeiweißstoffe, vor allem des Serumalbumin- und gamma-Globulinspiegels.

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UNTERSUCHUNGEN ÜBER DIE DAS POLIOMYELITIS-VIRUS
NEUTRALISIERENDE WIRKUNG VON SPEICHEL UND MAGENSAFT

Auf dem Kongreß 1961 der Ungarischen Mikrobiologischen Gesellschaft hatten wir mitgeteilt, daß der Magensaft von anaziden Individuen bakterielle Antikörper enthält. Anschließend untersuchten wir die virusneutralisierende Wirkung des Magensaftes anazider Personen.

Mit dem Serum, Speichel und Magensaft von 15 Personen, die an Achlorhydrie litten, wurde in der He-La-Zellkultur die Neutralisierungsprobe gegenüber Poliovirus 1, 2 und 3 vorgenommen. Wie festgestellt wurde, ist der Magensaft und Speichel von achlorhydrischen Individuen imstande, den zytopathogenen Effekt der untersuchten Viren zu neutralisieren. Der virusneutralisierende Titer des Speichels ist niedriger als der des Magensaftes, und der Antikörpergehalt beider hängt in einem gewissen Maße vom virusneutralisierenden Antikörperspiegel im Serum ab.

Nach den Ergebnissen der mit Magensaft und Speichel durchgeführten papier- und immunoelektrophoretischen Untersuchungen enthalten beide Flüssigkeiten gamma- und beta-Globulin. Zwischen dem Antikörperspiegel der untersuchten Säfte und ihrem Eiweißgehalt besteht eine Korrelation.

Die Frage der Zusammenhänge zwischen Globulinspiegel, Antikörpergehalt und mononukleärer Zellzahl studierten wir mittels zytologischer Untersuchung des Magensaftes.

Den lokalen Antikörpern kommt in der Abwehr der enteropathogenen Infektionen Bedeutung zu, und sie vermögen auch den Erfolg der Sabin-Schutzimpfung zu beeinflussen.

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UNTERSUCHUNG DER VIRUSAUSSCHIEDUNG UND ANTIKÖRPERBILDUNG
NACH IMMUNISIERUNG MIT LEBENDEM ATTENUIERTEM POLIOVIRUS
TYP I IN EINEM KINDERKOLLEKTIV IN SZEGED

Für die Untersuchungen wurden 2—9 Monate alte, demselben Kinderkollektiv angehörende 29 Säuglinge ausgewählt. Die Untersuchungen wurden in einer 3 Tage vor und 7 Tage nach der Impfung in 3tägigen Intervallen entnommenen Stuhlproben vorgenommen, in denen wir die Virusausscheidung und den Titer des ausgeschiedenen Virus bestimmten. Außerdem entnahmen wir dreimal eine Serumprobe: die erste 3 Tage vor der Vakzination, die zweite

und dritte 30 bzw. 120 Tage nach der Vakzination. Aus der vor der Vakzination entnommenen Stuhlprobe wurden in 6 Fällen andere Enteroviren (zum ECHO-Typus 14 und 11 gehörige Stämme) gezüchtet.

Die noch im Gange befindlichen Untersuchungen lassen den Schluß zu, daß es sich in etwa 8—10 Fällen bei dem im Stuhl ausgeschiedenen Virus gleichfalls nicht um das zur Vakzination benutzte attenuierte Polio-Virus, sondern um einen der erwähnten Enterovirus-Typen handelt. Entsprechend dem seltenen Anhaften des Poliovirus haben wir auch in den Serumproben selten (in 9 Fällen) signifikante Titererhöhung wahrgenommen.

Nach den Untersuchungsergebnissen waren in dem zur Auswertung der Vakzination ausgewählten Kinderkollektiv zur Zeit der Immunisierung eine ganze Reihe von Säuglingen symptomfreie ECHO-Virus-träger, wodurch das Anhaften des Virus in hohem Maße gehemmt wurde. Eine Stütze dieser Beobachtung bilden auch die Resultate unserer in der Periode vor der Vakzination vorgenommenen Reihenuntersuchungen an 122 Szege der Einwohner, bei denen in ziemlich hohem Verhältnis Enterovirus-Stämme desselben Typs gezüchtet wurden. Einige Personen waren mehrere Monate hindurch Virus-träger.

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UNTERSUCHUNG DER PERORALEN SH. FLEXNERI-VAKZINATION AN MÄUSEN

Bei aktiven Mäuseschutzversuchen kann peroral unter Anwendung einer genügenden Zahl von Stimuli und einer ausreichenden Antigenmenge eine der subkutan ausgeführten Vakzination äquivalente Immunität herbeigeführt werden. Peroral sind mindestens 2 Stimuli zur Erreichung einer bewertbaren Immunität nötig. Das Verhältnis der äquivalenten Antigenmengen beträgt bei der peroralen und subkutanen Methode etwa 100 : 1.

Im Rahmen passiver Mäuseschutzversuche untersuchten wir neben den Serum-Antikörpern auch die Copro- (Mucus-) Antikörper der Mäuse. Das Mäuseschutz-Titermaximum der Serumantikörper ist in der 2. Woche nach der Immunisierung zu beobachten, doch können noch in der 4. Woche mit beträchtlichem Titer, wenn auch mit sinkender Tendenz Antikörper nachgewiesen werden. Das Titermaximum der Copro-Antikörper trat früher, bereits in der ersten Woche zutage, während die Schutztiter in der 3. Woche schon wesentlich gesunken waren und in der 4. Woche nur in unbedeutender Menge demonstriert werden konnten.

In-vitro-Versuche (HaG) führten zu denselben Ergebnissen wie die passiven Mäuseschutzversuche. Die Antikörper (Agglutinine) zeigten sowohl im Serum wie im Mucus inkompletten Charakter.

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UNTERSUCHUNGEN
MIT DER ANTI-HUMAN-GLOBULIN-KONSUMPTIONSPROBE
BEI LEBERERKRANKUNGEN

Mit der zur Mikromethode umgestalteten Form des von STEFFEN beschriebenen Anti-Human-Globulin-Konsumptionstestes (AGCT) wurden bei 72 an verschiedenen Leber- und Gallenwegserkrankungen, 12 an Mischinfektion und 28 an anderen internistischen Erkrankungen leidenden sowie bei 29 gesunden Individuen Untersuchungen durchgeführt, wobei wir die Probe im Verlauf Krankenhausaufenthalts der Kranken 2—5mal wiederholten.

Der AGCT fiel in den langsam heilenden Fällen von akuter bzw. chronischer Hepatitis und Zirrhose zu 82%, bei den komplikationsfrei heilenden Hepatitiden zu 54%, bei den Gallenwegserkrankungen zu 31%, bei Mischinfektionen zu 75% und bei anderen inneren Erkrankungen zu 21% sowie bei den gesunden Personen zu 14% positiv aus. Bei der Auswertung der Ergebnisse betrachteten wir nur diejenigen Kranken als negativ reagierend, bei denen auch die Wiederholungen in keinem Fall positiv ausfielen.

Bei den positiv reagierenden, aber verzögerte Remission zeigenden Kranken mit akuter Hepatitis wurden anlässlich der Wiederholungen insgesamt 94 Untersuchungen durchgeführt, und in 37 dieser Fälle war die Reaktion negativ. Im allgemeinen wurde die positive Probe dann negativ, wenn den Kranken bei der Therapie Rindenhormone verabreicht worden waren. Im Verlauf der Prednisolon-Behandlung verzeichneten wir ein negatives Ergebnis in 64%, vor oder nach der Prednisolon-Behandlung in 33% der Fälle.

I. GÁBOR und A. POTONDI

(Institut für Gerichtliche Medizin der Medizinischen Universität, Budapest)

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METABOLIC ROLE OF OLIGOSACCHARIDES IN ALCOHOLIC FERMENTATION BY YEASTS

By

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(Received November 19, 1962)

Summary. In the anaerobic metabolism of yeasts the role of oligosaccharides formed during the acid hydrolysis of starch, varies with different species or variants. Of the examined cultures, baker's yeast utilized only mono- and disaccharides, while *Saccharomyces sake* fermented in addition trisaccharides. *Schizosaccharomyces pombe* was able to use also oligosaccharides of higher molecular weight. In starch hydrolysate medium inoculated with *Schizosaccharomyces pombe* two oligosaccharides absent from the original medium appeared during fermentation. The possible mechanism of the formation of these substances is outlined. It is also shown that di-, tri, and tetrasaccharides are not fermented directly, but through an intermediate resynthesis.

The role of oligosaccharides in the metabolism of yeasts considerably varies with different species or even variants. Despite of extensive research in recent years, the particularly interesting and practically important problem of the fermentability and assimilation of oligosaccharides is not yet perfectly solved. The present paper deals with the fermentability of oligosaccharides produced during the acid hydrolysis of starch. These substances and their identification by paper chromatography were described in a previous report [1]. On the basis of the R_f values it has been shown that the hydrolysate contains polymers consisting of 2, 3, 4, 5, 6 and 7 glucose molecules. As indicated by the colour reactions with p-amino phenol, these substances contained glucose units linked in the 1,4 position.

Acid hydrolysis of starch thus yielded a mixture of carbohydrates composed of glucose, the above oligosaccharides and dextrines. The latter contained more than 7 glucose molecules; they remained unidentified as they did not run on the chromatogram. The carbohydrate mixture supplemented with inorganic salts and bios factors was inoculated with *Saccharomyces cerevisiae*, *Saccharomyces sake* and *Schizosaccharomyces pombe* and the changes of carbohydrates were followed by paper chromatography. In some instances the reducing and total sugar content of the medium was also determined.

Materials and methods

Preparation of starch hydrolysate. In a 2 l beaker 200 g commercial potato starch was mixed at room temperature with 800 ml distilled water, by means of a glass rod. The starch was allowed to swell, then placed in the Höppler ultrathermostate filled with an aqueous glycerol bath of 100° C. After heating for 20 minutes, 32 ml concentrated hydrochloric acid

(analytical purity) was added to the gel. The mixture was continuously stirred until liquefaction was complete. After one hour the beaker was removed from the glycerol bath and quickly cooled under running water. The content of the beaker was then neutralized with granulated sodium hydroxide. Under these conditions only about 35 per cent of the starch was hydrolysed. Thus in addition to the end product of hydrolysis glucose, the different oligosaccharides were present in sufficient amounts to be detected chromatographically.

Medium containing oligosaccharides. To the neutralized hydrolysate 100 ml inorganic salt solution and 30 ml yeast autolysate were added, then the volume was brought up to 1000 ml. The reaction of the solution was checked and adjusted to pH 5. The medium was distributed in 100 ml amounts into 300 ml Erlenmeyer flasks and sterilized at 110° C for 1 hour on two consecutive days.

Inorganic salt solution. In 1 l distilled water 50 g KH_2PO_4 , 50 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.6 g $\text{Ca}_3(\text{PO}_4)_2$ and 1.0 g $(\text{NH}_4)_2\text{SO}_4$ were dissolved [2].

Yeast autolysate. One part of yeast cake was mixed with one part of water and the suspension was incubated for 48 hours at 48° C. Then the supernatant was separated by centrifugation, run through a cellulose filter, and sterilized at 110° C for 1 hour on two consecutive days. The autolysate prepared in this manner was free from deposits [2].

Maltose medium. One hundred grams of maltose (analytical purity), 100 ml inorganic salt solution and 30 ml yeast autolysate were mixed and the volume was brought up to 1000 ml. After an adjustment to pH 5, the medium was distributed in 100 ml amounts into 300 ml Erlenmeyer flasks and sterilized as described above.

Inoculation. The cultures were transferred into test tubes containing malt extract of 8 Balling degrees. The tubes were incubated at 30° C, or, in the case of *Schizosaccharomyces pombe*, at 37° C, for 24 hours. Then the medium was decanted and the deposit was used as inoculum for the starch hydrolysate or maltose medium.

Fermentation. Fermentation was carried out at 30° C for *Saccharomyces cerevisiae* and *sake*, and at 37° C for *Schizosaccharomyces pombe*. Samples were taken for chromatographic examination at 6–24 hour intervals under sterile conditions.

Paper chromatographic analysis of sugars. The mash samples were filtered through folder-filters, then freed from cations and anions with Dowex 50 and Dowex 1 ion exchange resins. The deionized solutions were concentrated to the original volume by evaporation. With two subsequent spottings, 0.6 μl aliquots of the solutions were placed on Schleicher-Schuell No. 2043/a filter paper, on a straight line drawn at 8 cm distance from the edge of the paper sheet. The spots were spaced at 2.5 to 3 cm intervals. Descending chromatograms were run at room temperature by the "Durchlauf" technique [3]. The length of time of development was 4 days for the separation of mono- and disaccharides or 6 days for the separation of oligosaccharides with 3–7 glucose molecules. As a solvent butanol–acetic acid–benzol–water (5 : 3 : 1 : 3) was used [4]. After development the paper sheet was dried at room temperature, then freed from the solvent by exposure to 100° C. The sugars were detected by spraying with p-amino phenol reagent prepared as follows. One gram of p-amino phenol was thoroughly mixed with 4 ml concentrated phosphoric acid, dissolved in 100 ml 96 per cent ethanol and finally filtered through a folder-filter. After spraying the chromatograms were placed for 5 minutes in a 100–105° C oven. Glucose polymers linked in the 1,4 position gave a yellow, those in the 1,6 position a brown, colour reaction on sandy background [5]. Glucose and maltose were identified by means of simultaneously run control solutions. Oligosaccharides of higher molecular weight were recognized on the basis of the correlations between the number of molecules constituting the polymer homologues and R_f or R_M values [6, 7].

The reducing and total sugar content of the media was determined by BERTRAND's method [8].

Results

The paper chromatographic examination of completely fermented mashes showed that *Saccharomyces cerevisiae* utilized only glucose and maltose, while *Saccharomyces sake* fermented in addition maltotriose (Fig. 1). This finding may be due to the fact that baker's yeast is habitually grown in media containing plenty of fermentable mono- and disaccharides, but only one trisaccharide (raffinose) in small quantities. Raffinose, being composed of glucose, fructose

and galactose, principally differs from the only glucose-containing oligosaccharides produced during the hydrolysis of starch. In contrast, the routine medium for *Saccharomyces sake* consists of such glucose-containing oligosaccharides. The fermentation of maltotriose as a property varying with different strains within the species or variant, was described by GILLILAND [9]. It should be

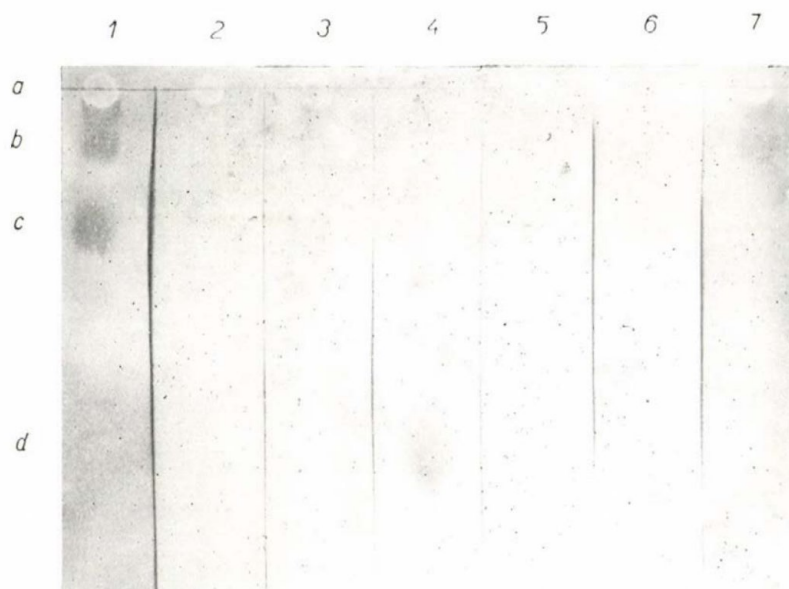


Fig. 1. Fermentation by different yeasts of starch hydrolysate containing oligosaccharides 1: *Schizosaccharomyces pombe*, 48 hours fermentation. — 2: *Schizosaccharomyces pombe*, 52 hours fermentation. — 3: Baker's yeast, fermentation complete. — 4: Maltose control. — 5 and 7: *Saccharomyces sake*. 6: Fermentation of maltose by *Saccharomyces sake* — a and b: Oligosaccharides containing more than three molecules of glucose. — c: Trisaccharide. — d: Maltose

noted that we were unable to adapt baker's yeast to oligosaccharides by serial transfers in a medium containing these substances.

Schizosaccharomyces pombe is known to ferment oligosaccharides. In a previous paper [1] it has been reported that during fermentation by this yeast, two oligosaccharides differing in both R_f value and colour reaction from the originally present substances, can be observed. As fermentation proceeds, these as well as the other oligosaccharides disappear gradually from the medium. Fig. 2 shows that in the completely fermented mash, of the oligosaccharides present in the uninoculated medium, only the heptasaccharide can be detected. On the basis of the colour reaction the two intermediate oligosaccharides were considered as 1,6-glucose polymers. From the quantitative changes in the carbohydrate content during fermentation it was concluded that these polymers might be formed by transglucosidation from the originally present 1,4-tri- and tetrasaccharides.

Japanese workers [10—13] found that *Schizosaccharomyces pombe* or its enzyme-containing extract produces from maltose isomaltose, glucose, sakebiose, kojibiose, panose and dextrantriose. On the basis of our chromatograms it seemed possible that *Schizosaccharomyces pombe* synthesized the two

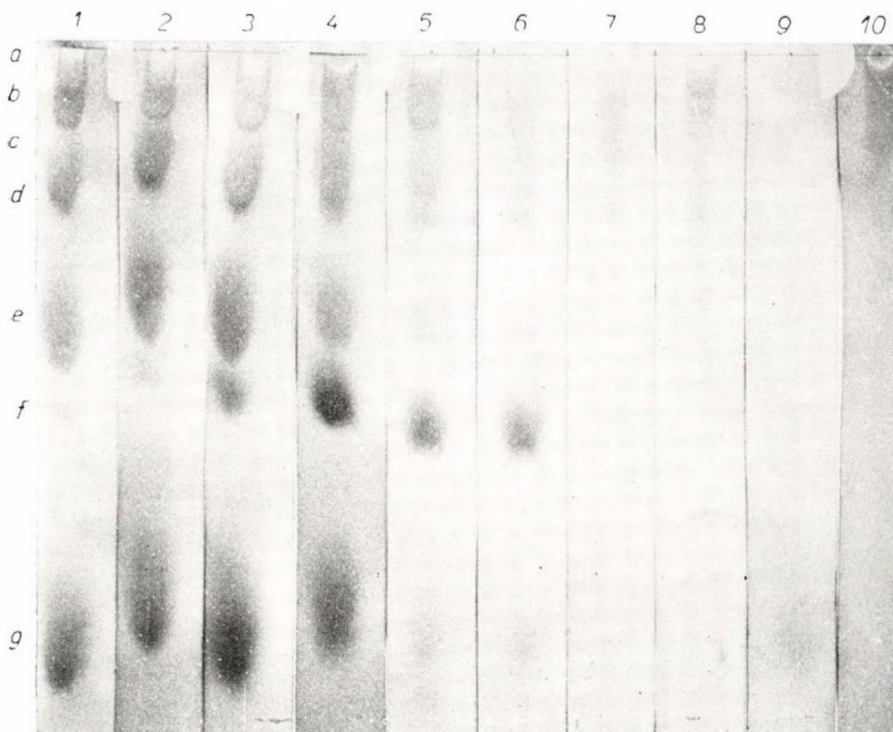


Fig. 2. Fermentation of starch hydrolysate by *Schizosaccharomyces pombe*. Samples taken after 0 (1), 3 (2), 6 (3), 12 (4), 18 (5), 24 (6), 36 (7), 48 (8) and 60 (10) hours. 9: Maltose control a: Hexasaccharide. — b: Pentasaccharide. — c: Tetrasaccharide, originally present in the medium. — d and f: Oligosaccharides produced during fermentation. — e: Trisaccharide (maltotriose) originally present in the medium. — g: Maltose

1,6-oligosaccharides from maltose. In experiments with maltose-containing medium seeded with that organism glucose and two other sugars appeared shortly after inoculation (Fig. 3). Of the latter the one with a higher R_f value was produced in much larger amounts than the other with a lower R_f value. The amount of these substances reached a maximum at the beginning of fermentation, then decreased and finally, at the end of the process, disappeared. On the basis of R_f values and colour reaction, the two sugars synthesized from maltose were identical with the 1,6-oligosaccharides produced during the fermentation of starch hydrolysate.

In contrast to the four synthesized oligosaccharides described by the Japanese authors, in our experiments we found only the indicated substances.

The difference in the findings may be attributed to different enzyme systems in the used *Schizosaccharomyces pombe* strains. Accordingly, it may be assumed that the ability to synthesize oligosaccharides is a variable property of strains belonging to this species. After our first paper had been published [1], PHILLIPS [14] reported that during the fermentation of wort by *Schizosaccharomyces pombe* two 1,6-oligosaccharides were only produced.

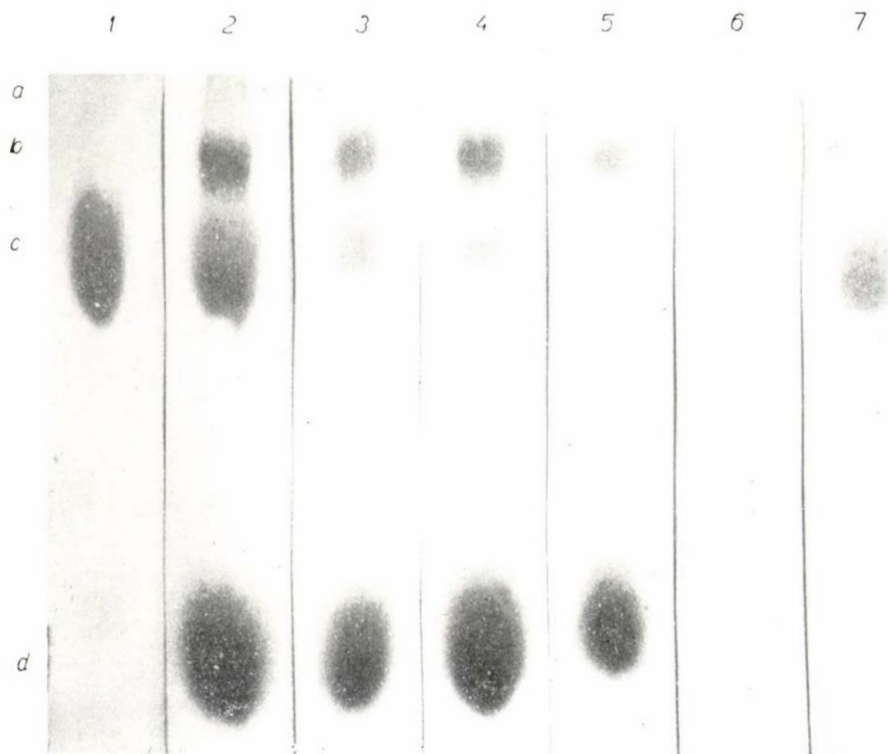


Fig. 3. Fermentation of maltose by *Schizosaccharomyces pombe*. Samples taken after 0 (1), 4 (2), 8 (3), 12 (4), 16 (5), 32 (6), hours. 7: Maltose control. — a and b: Oligosaccharides produced during fermentation. — c: Maltose. — d: Glucose

In subsequent experiments it was studied whether in maltose-containing starch hydrolysate *Schizosaccharomyces pombe* was able to produce 1,6-oligosaccharides also from 1,4-tri-, or tetrasaccharides as well as from maltose. For these examinations a maltose-free medium containing 1,4-tri- and tetrasaccharide was needed. In order to remove mono- and disaccharides, the starch hydrolysate medium was inoculated with baker's yeast (Fig. 4). The chromatogram clearly shows that after fermentation had been completed, the mash was free from glucose and maltose and contained only 1,4-oligosaccharides. From the mash yeast cells were filtered out and after sterilization the filtrate was seeded with *Schizosaccharomyces pombe*. The mash was sampled at suitable

intervals for chromatographic analysis and for the determination of reducing and total sugar content.

In the first hours following inoculation the concentration of total reducing substances was unchanged, while that of directly reducing substances in-

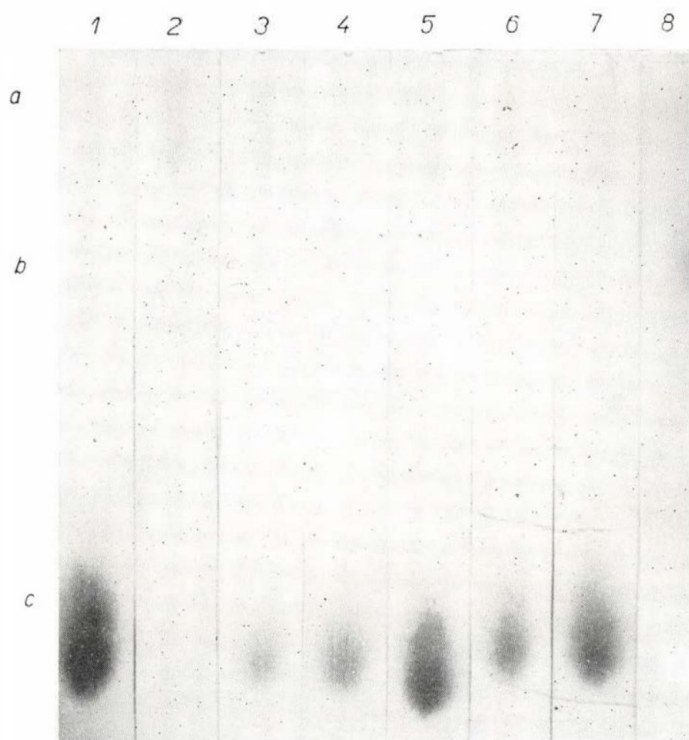


Fig. 4. Fermentation by *Schizosaccharomyces pombe* of starch hydrolysate containing only oligosaccharides. — a: Oligosaccharides. — b: Maltose. — c: Glucose. — 1: Original starch hydrolysate. 2: Starch hydrolysate fermented by *Saccharomyces cerevisiae*. 3—7: Fermentation by *Schizosaccharomyces pombe* of oligosaccharides in starch hydrolysate pre-fermented with *Saccharomyces cerevisiae* 8: Maltose control

creased. This finding indicated that although the enzymic decomposition of carbo-hydrates had started, fermentation did not take place during that period.

The chromatogram (Fig. 4) showed that after inoculation with *Schizosaccharomyces pombe*, glucose, maltose and two 1,6-oligosaccharides appeared in the mash containing originally only 1,4-oligosaccharides.

Discussion

The question whether the two different 1,6-oligosaccharides produced by *Schizosaccharomyces pombe* during the fermentation of starch hydrolysate are formed from maltose or from 1,4-tri- and tetrasaccharides, has not been definitely

answered by the results of the present experiments. The production of these substances may be explained by two mechanisms. The originally present 1,4-oligosaccharides may be converted partly into glucose and maltose, and partly, by transglucosidation, into 1,6-oligosaccharides. It should be noted that WILLSTÄTTER *et al.* [15] pointed out the possibility that under certain conditions in maltose fermentation, instead of the production of two glucose molecules by maltase, transglucosidation and phosphorolysis occur. However, it seems more likely that the yeast hydrolyses the original tri- and tetrasaccharide to maltose and glucose, and synthesizes 1,6-oligosaccharides from maltose. This is supported by the finding that *Schizosaccharomyces pombe* also forms 1,6-oligosaccharides in the medium containing only maltose.

Accordingly, the originally present 1,4-tri- and tetrasaccharides and maltose are probably fermented or assimilated by this organism through a resynthesis of 1,6-oligosaccharides. No explanation has been offered by the experiments as to whether the fermentation of the other hydrolytic products of starch, the 5 or 6 glucose molecule-containing oligosaccharides, takes place in this manner.

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INFLUENCE OF TEMPERATURE ON THE MULTIPLICATION OF VARICELLA VIRUS

By

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(Received December 27, 1962)

Summary. Reproduction of the varicella virus was studied in human fibroblastic cultures at 30°, 37° and 39° C.

1. The time necessary for the development of continuous monolayer was approximately the same at 39° and 37° C; at 30° C the process was considerably slower.

2. For the reproduction of the varicella virus 37° C is the optimal temperature. The virus synthesis is remarkably lower at 30° C and stops at 39° C.

3. At 39° C the initial (intranuclear) phase of the reproduction is inhibited. The intracellular virus remains infectious at this temperature for at least seven days.

4. The slower rate of virus reproduction at 30° C may be due to the reduced cell metabolism at lower temperatures.

Virus multiplication is highly sensitive to temperature. In general, temperatures around 37° C are held to be most favourable for reproduction.

According to WELLER [1, 2], TAYLOR-ROBINSON and DOWNIE [3] and VÁCZI *et al.* [4] the varicella virus, unlike other viruses, cannot be detected in the medium of the infected tissue culture; its passage needs cell suspension as inoculum. This fact considerably hinders the laboratory study of the varicella virus. For this reason efforts have been made in this Institute to obtain extracellular infectious virus. In the course of these studies the influence of certain conditions on the multiplication of the virus have been examined. The present study is concerned with the influence of the temperature.

Materials and methods

Virus. The strain of varicella virus used in the experiments was isolated [4] and maintained in human embryonic fibroblastic cultures. The strain had undergone 30–46 passages. The nutrient fluid contained 80 per cent HANKS' balanced solution with 0.5 per cent lactalbumin hydrolysate and 0.1 per cent NaHCO_3 , and 20 per cent bovine serum. As indicator, phenol red was employed.

The cell cultures were inoculated with 0.2 ml of infected cell suspension. The inoculum was prepared from human fibroblastic cultures infected 8–10 days previously.

Preparation of infectious cell suspensions. Monolayers were grown in 16 × 110 mm tubes. Cell suspensions were prepared from three cultures each by treatment with 0.25 per cent solution of Difco trypsin. The cells were sedimented by centrifugation at 1000 r. p. m. and resuspended in 2 ml medium. The tube cultures were inoculated with 0.2 ml of the 1 : 100-diluted cell suspension.

Preparations of fibroblast cultures. Boned human embryos 1–3 months of age were minced and digested with 0.15 per cent trypsin at 4° C overnight. The cells were settled by centrifugation and re-suspended in the nutrient medium. Fifteen million cells were placed

in each of Roux flasks. As the monolayer had developed, the cells were removed by trypsinization. Of the cell suspension obtained by dilution in medium 1.5 ml containing 300,000 cells were placed in each of culture tubes; the cultures were incubated at 37° C. Monolayers suitable for inoculation developed by the 2nd–4th days of incubation.

Cover-slip cultures. Two cover slips of 18 × 18 mm were placed in each of flasks with a basic area of 8 cm² and 800,000 fibroblastic cells were added in 2 ml nutrient medium. The cultures were incubated at 37° C.

Virus multiplication was measured on the basis of the number and two-dimension size of the foci appearing in the cultures. To estimate the growth of foci, these were projected and plotted every day and the plotted areas were measured by planimetry.

Results

Virus reproduction was studied at 30°, 37°, and 39° C. Since the virus multiplies only in active, well-metabolizing cells, first multiplication of the fibroblasts was studied.

The cell suspension was distributed in tubes and these were incubated at 30°, 37° and 39° C. The time necessary for formation of continuous monolayer was registered, and the monolayers were stained with haematoxylin and eosin to observe possible cell degeneration.

At 37° or 39° C the formation of complete monolayers took two days; at 30° C four days were necessary. At 39° C the cells were larger and more elongated and the cell count was lower than at 37° C.

Reproduction of the varicella virus. Four-day-old monolayers were inoculated each with 0.2 ml of 1 : 100-diluted cell suspension obtained from human fibroblast cultures that had been infected 8–10 days previously. Cultures were incubated at 30°, 37° and 39° C, and examined daily for cytopathic foci; these were counted and the results were plotted against time (Fig. 1).

In unstained cultures the first foci were observable on the third day after inoculation. The number of foci had been rising up to the 8th day; then because of confluence of foci, degeneration became diffuse.

At 30° C changes required three more days to appear. After eight days of incubation 45 foci were counted in contrast with the 75 observed in the parallel culture incubated at 37° C. Confluence of foci appeared on the 14th day.

At the initial stage the foci developing at 30° C were morphologically different from those growing at 37° C; the rounded cells were less refractile, thus less easily distinguishable from the neighbouring intact cells.

At 39° C cytopathic changes appeared only in one of the four experiments, on the sixth day after inoculation. After a very slight rise in number the process stopped spontaneously; the affected cells were destroyed completely and detached. Subsequently the discontinuity of the monolayer was substituted by new fibroblasts, the monolayer was thus restituted.

The increase of the foci was determined daily by measuring 10 foci developing at each of the above three temperatures. The data given in Fig. 2 are average values.

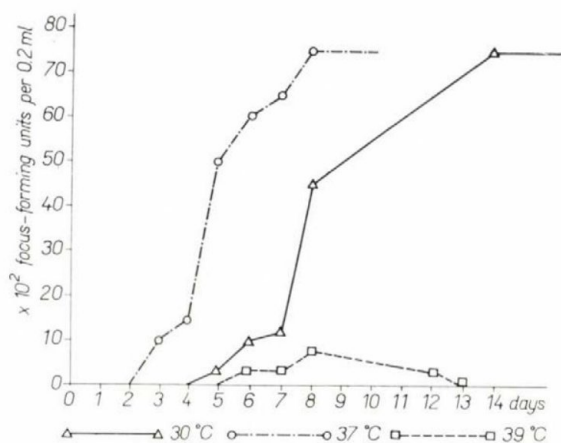


Fig. 1. The number of countable foci on days after infection

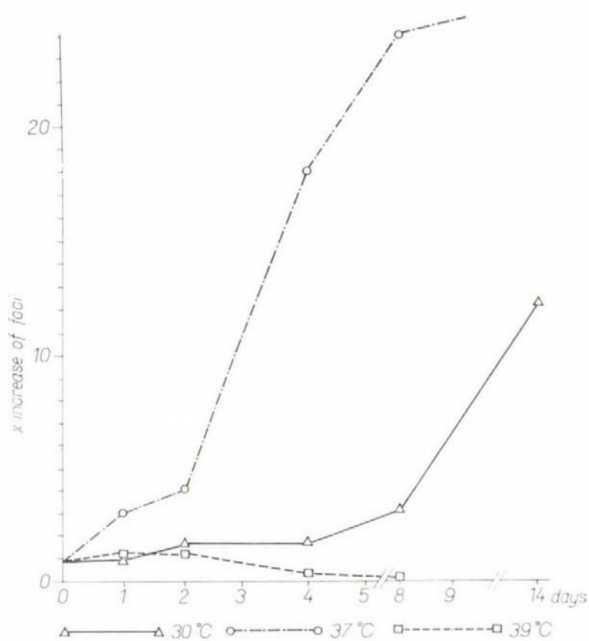


Fig. 2. Growth curves of cytopathic foci in cultures incubated at different temperatures after pre-incubation three days at 37°C; each plot represents the average area of ten foci

It is clearly seen that the increase of cytopathic foci was the most rapid at 37°C; during the period from the fourth through the 12th day after inoculation a 24-fold increase of the average area was registered. The process was considerably slower at 30°C (12-fold growth by the 14th day). In the cultures grown

at 39° C a very slight growth (1.1-fold, on the average) was observed after 24 hours. Then the lesions stopped growing, even began to decrease (to 0.3 units) and, at last, they disappeared being replaced by a new monolayer consisting of new fibroblasts.



Fig. 3. Cytopathic lesion in cultures incubated after infection with varicella virus at 37° C for 24 hours and, subsequently, at 39° C for two days. Magnification approx. $\times 400$

Fig. 3 and 4 show the same focus at two stages. The monolayer developing the focus was incubated at 37° C for 24 hours, then placed in an incubator of 39° C; at this time foci were not observable in the monolayer. Foci appeared after another 24 hours (Fig. 3) and increased considerably slower than those incubated at 37° C. Subsequently the centre of the focus detached from the glass wall while its shrinking peripheral cells formed a narrow wreath-like border sharply distinct from the neighbouring normal cells (Fig. 4). Finally, the degenerated cells were completely destroyed, cells grew from the intact part of the monolayer, under the debris, towards the centre and developed a new monolayer covering the site of the focus.

In further experiments cell cultures were inoculated with varicella-virus infected cells, and the cultures were incubated at 39° C for seven days. Cytopathic changes did not develop at this temperature. Subsequently, however, the cultures were placed in an incubator of 37° C. Cytopathic changes soon appeared showing that the inoculum cells had not lost their infectiousness during seven days at 39° C.

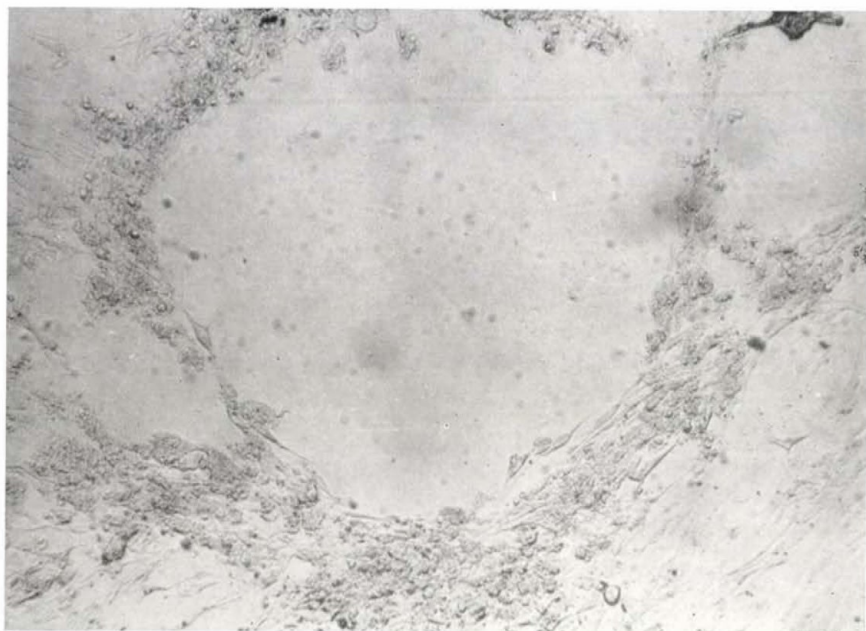


Fig. 4. The same lesion as in Fig. 3, after six days of incubation at 39° C

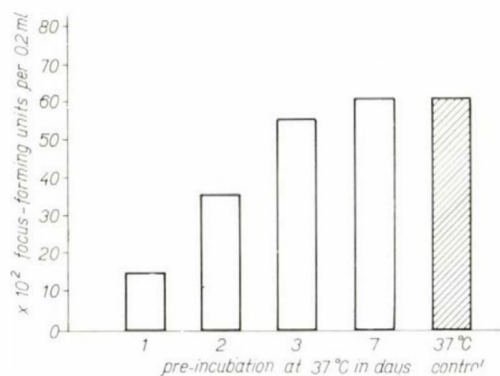


Fig. 5. Numbers of countable foci in cultures incubated after infection at 37° C for varying periods of time, and subsequently at 39° C for 3 days

Finally, we examined the influence of the time of pre-incubation at 37° C on the appearance of foci during subsequent incubation at 39° C (Fig. 5).

If temperature was changed for 39° C before the highest plaque count had been attained, the final number of foci remained below that of the control cultures kept at continuously 37° C. The fate of the plaques which had developed was the same as of the others grown at 39° C.

Discussion

Numerous authors have studied the influence of temperature on virus multiplication. According to LEWIS and OVERMAN [5], who studied the vaccinia virus, the virus yield was 200—3000 times less at 26° C than at 37° C. Primarily the number of non-infective particles increased on the account of the infective ones. Consequently, cytopathic changes were less prominent at 26° C than at 37° C.

According to WHEELER and CANBY [6] herpes simplex virus does not propagate at 26° C; it remains instead in a masked state inside the cell. Such cultures begin synthesizing virus when placed in adequate temperatures. It is assumed that the viral or cellular nucleic-acid production is reduced at lower temperatures. HOGGAN and ROIZMAN [7] also demonstrated that the reproduction of herpes simplex virus and the appearance of free herpes virus are highly dependent on temperature. The accumulation of virus in the cell was the greatest at 34° C. Extracellular virus hardly appeared at this temperature; latter reached the highest value at 37° C; the poorest yield was observed at 39° C.

It is a peculiarity of the temperature—virus relation that mutants with high and low pathogenicity can be distinguished from each other by cultivation at various temperatures (*e.g.* poliovirus [8], Aujeszky disease virus [9]). Certain plant viruses can also be distinguished from each other by examining their reproduction at different temperatures [10].

Regarding the peculiar reproduction of the varicella virus, it seemed to be of interest to study the reproductive capacity of this virus at various temperatures. For the reproduction of human fibroblastic cells, 37° C proved to be most favourable among the three temperatures studied. At 30° C their multiplication is slower as shown by the delayed monolayer formation. In spite of this, approximately the same cell count can be reached at both temperatures. At 39° C monolayers develop as soon as at 37° C, but the cells are enlarged and fewer cells can be recovered by trypsinization.

Optimal virus reproduction was observed at 37° C; at 30° C the yield was nearly the same, but the rate of multiplication was lower. In contrast, no virus multiplication was observed in most of the cultures incubated at 39° C. If a few foci appeared, these were atypical and soon regressed spontaneously. Extracellular virus could not be obtained, whichever temperature was applied.

Varicella-virus-infected cells may keep the infectious virus for at least seven days when placed on cultures incubated at 39° C. Such cultures develop foci after changing the temperature for 37° C.

After incubation of infected cultures at 37° C the foci that had already appeared kept growing after changing temperature for 39° C. Even new foci appeared at this temperature. The final number of foci was in direct relation

to the period of incubation at 37° C. However, the foci soon began to decrease at 39° C and not much later disappeared showing that virus reproduction was considerably impaired at that temperature.

By means of the immunofluorescent technique we succeeded in detecting viral antigen in the nuclei of infected cells as soon as 24 hours after inoculation, *i.e.* at a time when no cytopathic changes had yet appeared. However, virus reproduction stopped as soon as the temperature was changed for 39° C. The cells having produced intranuclear virus antigen during incubation at 37° C developed cytopathic changes during subsequent incubation at 39° C, showing that virus synthesis was continuous even at this temperature. Accordingly, only the intranuclear phase of virus synthesis was inhibited by the elevated temperature. The fact that a reduced cell count and enlarged cells were observed even in non-infected cultures at 39° C suggests that nucleic-acid synthesis is hindered even in the normal fibroblasts at this temperature. As a result of this, the protein/nucleic-acid ratio is thought to be changed in the cells.

We suppose that similar changes might be responsible for the cessation of varicella virus reproduction at 39° C.

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EFFECT OF TEMPERATURE ON THE POTENTIOMETRIC TITRATION CURVE OF INFLUENZA VIRUS

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Summary. Analysis of the potentiometric titration curves obtained at different temperatures for 1 g influenza virus protein N has shown that the ionizing groups of this virus may be divided into three classes on the basis of the apparent heats of ionization (Q').

Class 3 consists of uniform groups with an average Q' value of +10,300 calories and pK'_1 9.75. These groups are most probably the ϵ -NH₂ residues of lysine.

Groups of class 2 have an average Q' value of +6,400 calories and a pK' of 6.97, thus they are very likely the imidazolium residues of histidine.

All other groups, *i.e.* those in the acid part or the transition zone between Class 1 and Class 2 and those in Class 1, have Q' values between +2000 and +3000 calories, therefore they might be regarded as carboxyls.

The thermal method of analysis yielded pK' values for influenza virus within the range of pH 3.00 to pH 10.75 as follows: $pK'_1 = 3.50$; $pK'_2 = 4.50$; $pK'_3 = 5.11$ (5.125); $pK'_4 = 5.73$; $pK'_5 = 6.97$; $pK'_6 = 9.75$.

The values for acid or base bound by the individual groups agreed well with similar data obtained earlier.

No terminal amino or carboxyl groups were found to ionize.

In a previous paper a report was presented on the potentiometric titration of the ionizing groups of influenza virus [1]. The characterization of the titratable groups was attempted on the basis of changes observed on treatment with formaldehyde.

The present paper contains our recent results obtained by analysing the titration data yielded by the "thermal method" of WYMAN [2].

Materials and methods

As the materials and methods used in the present study were essentially the same as those used previously [1], we refer here only to some slight modifications.

Purification of the virus. The eluate (Paris PL 1/49 A' virus) obtained after adsorption to and elution from triple washed chicken red blood cells was purified by alternate cycles of high and low speed centrifugation, essentially as described by STANLEY [3]. The haemagglutination unit per mg N ratio of this preparation was the same as that obtained by others (5×10^5).

Potentiometry. The solutions and experimental conditions were identical to those used previously, except that triplicate titrations were performed at three different temperatures, *i.e.* at $+10^\circ \pm 0.1^\circ$ C, at $+25^\circ \pm 0.1^\circ$ C and at $+37^\circ \pm 0.1^\circ$ C. The temperature required was obtained by keeping the titration container in an appropriate water bath. Titrations were begun 5–10 minutes after the temperature had been stabilized. The apparatus was the same Metrohm Herisau Type E 166 Titriscope that had been used earlier. The electrode, however, was in the present study a Metrohm "U" microelectrode which has a smaller alkaline error than the previously used Metrohm "X" microelectrode.

Calculation of the results. From the mean values (reduced to 1 mg protein N) of the three parallel titrations at each temperature the values of $\bar{h}$ were calculated for each pH, taking the $\bar{h}$ values at 25° C as reference data. In present experiments the value for n was 0.69, in good agreement with that obtained earlier (0.688).

Titration curves were plotted as values of $n - \bar{h}$ against pH. The values of pH shifts caused by titrating at temperatures different from 25° $\pm$ 0.1° C were obtained from the curves geometrically. The apparent heats of ionization (Q') were calculated by VAN't HOFF's equation (1), as described by WYMAN [2],

$$Q' = -4.579 T_1 T_2 \frac{pH_2 - pH_1}{T_2 - T_1}, \quad (1)$$

where T is the absolute temperature and the subscripts refer to the different experimental temperatures in question, taking always +25° C (298 T) as reference.

Experimental

Potentiometric titrations were performed by the method described previously [1]. Both the triplicate virus-free and the triplicate virus containing titrations were performed at three different temperatures given above. Titration data were calculated as the differences in the amount of 10^{-2} M HCl (in 0.137 M NaCl) required to reach a given pH in the presence

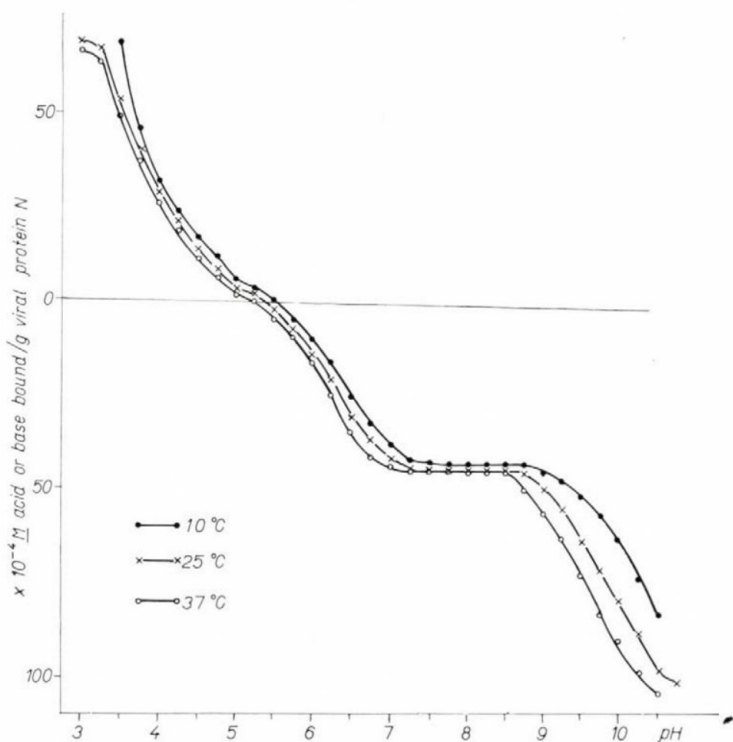


Fig. 1. Titration curves obtained for 1 g viral protein N at different temperatures

Table I*Values of $n-\bar{h}$ obtained at different temperatures for 1 mg viral protein N*

pH	$n - \bar{h}^*$		
	10° C	25° C	37° C
.75	.	-1.010	.
.50	-0.822	-0.975	-1.040
.25	-0.735	-0.880	-0.986
10.	-0.643	-0.792	-0.903
.75	-0.570	-0.715	-0.825
.50	-0.519	-0.636	-0.723
.25	-0.475	-0.550	-0.632
9.	-0.460	-0.498	-0.563
.75	-0.445	-0.460	-0.506
.50	-0.440	-0.458	-0.460
.25	-0.443	-0.458	-0.456
8.	-0.436	-0.462	-0.458
.75	-0.430	-0.462	-0.446
.50	-0.428	-0.458	-0.450
.25	-0.415	-0.440	-0.442
7.	-0.382	-0.420	-0.438
.75	-0.326	-0.366	-0.409
.50	-0.255	-0.320	-0.345
.25	-0.166	-0.210	-0.250
6.	-0.100	-0.140	-0.165
.75	-0.054	-0.074	-0.103
.50	+0.001	-0.020	-0.046
.25	+0.033	+0.020	-0.005
5.	+0.056	+0.030	+0.016
.75	+0.120	+0.080	+0.057
.50	+0.168	+0.140	+0.115
.25	+0.245	+0.210	+0.190
4.	+0.320	+0.290	+0.262
.75	+0.462	+0.405	+0.373
.50	+0.687	+0.545	+0.489
.25	.	+0.672	+0.635
3.	.	+0.690	+0.663

* The values are given in ml of 10^{-2} M HCl (in 0.137 M NaCl)

and in the absence of virus at each respective temperature. The numerical values for $n-\bar{h}$ obtained for 1 mg viral N are tabulated in Table I. The values found at 25° C were in good agreement with those observed previously.

Titration curves obtained at three different temperatures were plotted in the same coordinate (as presented in Fig. 1), taking the values of pH determined at 25° C as reference. The shifts in pH caused by titrating at lower or higher temperature were directly taken from the curves.

For the proper evaluation of the results we constructed a curve (see Fig. 2) by plotting values of Q' against the corresponding mean pH $\left(\frac{\text{pH}_2 + \text{pH}_1}{2}\right)$, as suggested by WYMAN [2]. The analysis of this curve showed that the ionizing groups of influenza virus could be divided into 3 classes on the basis of their apparent heats of ionization. Class 1 was characterized by ionization heats of +2000 to +3000 calories, class 2 by those of +6200 to +6700 calories, and class 3 by those of +10,000 to +10,600 calories.

There were two transition zones between the three classes. Of these the transition zone on the alkaline side showed a rather sudden decline after pH 9.25. This suggested a wide separation (2 pH units or more) of the pK' values of the most acid group of Class 3 and of that of the most alkaline group of Class 2. The midpoint of this transition zone, though poorly defined, appeared to be near pH 8.75. This means that the pK' of the most acid group of Class 3 could not have been less than about 9.75 and the most alkaline group of Class 2 not more than about 7.75. Thus one might consider the members of Class 3 to have no influence on the pK' values of the groups of the other classes.

The transition zone between Class 1 and Class 2 covered almost 2.0 pH units. Accordingly, the separation of the pK' values of the most acid group of Class 2 must have been less than 2 units. As deduced from the theoretical data presented by WYMAN [2], it appeared highly probable that the separation of the two nearest pK' values in the transition zone between Classes 1 and 2 should be about 1 unit. The midpoint of the transition zone was at about pH 6.40 to 6.50 (mean 6.45), *i.e.* the pK' value of the most alkaline group of Class 1 could not have been more than 5.90 to 6.00 and that of the most acid group of Class 2 not less than 6.90 to 7.00. From these facts and the corresponding theoretical considerations one should expect an effect of groups of Class 1 and Class 2 on each other's dissociation.

The analysis of the titration curve at +25° C in the light of the above data led to the following conclusions.

Members of Class 3 are uniform groups with a pK' of 9.75 and an apparent average heat of ionization of +10,300 cal. These data are fairly characteristic of the ϵ -amino group of lysine. As the ionization of these groups is not influenced by any other closely spaced group, the amount of base bound can be read

directly from the titration data and is 55×10^{-4} M/g virus N. This is in good agreement with the value (53×10^{-4} M/g virus N) obtained previously.

The base binding capacity of the most acid group of Class 2 and the most alkaline group of Class 1 is somewhat overlapping. From the data presented it seems to be probable that the overlap is approximately symmetrical, thus one may reasonably take the midpoint of the transition zone in Fig. 2 (about pH 6.45; base bound 29.0×10^{-4} M/g N) as the most probable endpoint of the titration of the most acid group of Class 2. The latter has an average Q' value of +6,400 calories, suggesting the presence of the imidazole residues of

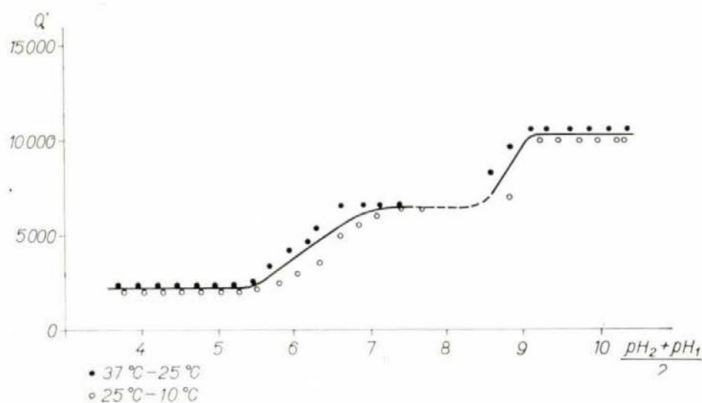


Fig. 2. Q' versus mean pH at different temperatures

histidine. Groups characterized by the same Q' value begin to dissociate at about pH 7.50, as demonstrated by the titration data obtained at 25° C. Thus the value of pK' is 6.97 for these groups. One may conclude, therefore, that the amount of base bound by the imidazole group is about 16.8×10^{-4} M/g virus N. This figure fits well to that (16.8×10^{-4} M/g virus N) calculated on the basis of certain theoretical considerations from the data of our earlier experiment.

The most alkaline group of Class 1 begins to dissociate at pH 6.45 and, as revealed by the titration data at 25° C, it binds base up to about pH 5.00. The pK' of this group is 5.73. The average heat of ionization for this group was +3.160 calories, roughly identical with that for the carboxyles. The total amount of base bound by this group is 32.0×10^{-4} M/g virus N, very similar to the value (31.9×10^{-4} M/g virus N) obtained earlier.

The acid group of Class 1, having an average heat of ionization of 2,200 calories, begins to dissociate at about pH 5.00 and takes up protons until pH 4.00 has been reached. The pK' for this set of groups is 4.50 and the amount of the acid bound, 26×10^{-4} M/g virus N.

The most acid group of Class 1, having an average heat of ionization of 2,200 calories, takes up protons between pH 4.00 and pH 3.00, thus having a pK' of 3.50. It binds a total of 40×10^{-4} M acid/g virus N.

From the data presented it is clear that there must be remarkable overlappings between the three groups of Class 1, too. An extensive interaction between the closely spaced groups should accordingly be supposed. Out of these interactions the one between the most alkaline and the acid groups of Class 1 is of utmost importance as this seems to be right in the environment of the pI determined by another method [4].

For convenience's sake we recall here the pK' values found, beginning with the acid range: $pK'_1 = 3.50$; $pK'_2 = 4.50$; $pK'_3 = 5.73$; $pK'_4 = 6.97$; $pK'_5 = 9.75$. It is clear that for groups with pK'_2 and pK'_3 there is overlapping between pH 4.73 and pH 5.50. This covers a total of about 10×10^{-4} M acid binding per g virus N. This value appears to be large enough to justify the supposition of the existence of a virtual pK' (pK'') resulting from the simultaneous dissociation of the overlapping groups. The value for this pK'' turns out to be 5.11. The existence of such a virtual pK' is made probable also by the fact that two clearly demonstrable and well reproducible inflexion points were regularly found at pH 5.00 and 5.25 in every titration curve. If we regard these two inflexion points as limits for a separately dissociating set of groups (acid bound 1×10^{-4} M/g virus N), we obtain a pK' of 5.125 for them. This value is very close to that deduced by the other method. Therefore it seems to be highly justified to regard pK'' as an actually existing value in the titration curve of influenza virus. Thus the final list of the apparent dissociation constants characteristic of the influenza virus preparation used, is $pK'_1 = 3.50$; $pK'_2 = 4.50$; $pK'_3 = 5.11$ (5.125); $pK'_4 = 5.73$; $pK'_5 = 6.97$ and $pK'_6 = 9.75$. The value of pI calculated by the generally accepted approximation formula [1] is 5.42 or 5.43, depending on the value attributed to pK'_3 . Both figures are very close to that (5.43) found by the rough approximation applied previously.

Discussion

The improvements achieved by applying WYMAN's "thermal method" [2] to the analysis of the titration curve of influenza virus were the successful separation of the hitherto confluent pK' values and the satisfactory physico-chemical identification of the groups involved.

In our previous study we could not identify pK'_3 , pK'_4 and pK'_5 found in present study. The "mixed" pK' of 6.37 in our earlier report turned out to have represented the mean value for pK'_4 and pK'_5 (mean: 6.35) yielded by the thermal method. The existence of pK'_3 has not been revealed at that time as there were no sufficient data available for a reasonable deduction. In the calculation of the pI by the approximation formula, the poor identification

of the individual closely spaced pK' values involved and the use of adequate mean values appears to have caused but little difference. This fact shows the role of the very extensive interactions between the closely spaced ionizing groups in determining the point of isoionic state and appears to justify the use of the approximation formula in cases where only mean values for some closely spaced pK' -s are available in the environment of the pI.

The moderate difference in the numerical values of pK'_1 found now and earlier should be ascribed to the experimental error involved in the determination of the extremely low differences in acid binding at the titration endpoint.

As to the probable nature of the ionizing groups detected up to now in the influenza virus, there is very little doubt left. This is particularly true for the ϵ -amino and the imidazole groups, which now might both be regarded as satisfactorily identified.

The carboxyl nature of the other groups found is highly probable, as all the acid pK' values found belong to the known range of apparent dissociation constants of glutamyl and aspartyl carboxyls, and the heats of ionization determined for these groups were also characteristic of carboxyls. We do not think, however, that we have any reliable data available to decide which of the groups were glutamyl- and which aspartyl carboxyls.

The data presented above do not suggest that any terminal carboxyl or amino groups would ionize in the influenza virus.

The value for the maximum acid binding has been found to be $70.4 \pm 1.4 \times 10^{-4}$ M/g viral protein N, while that for maximum base binding, $99.5 \pm 1.5 \times 10^{-4}$ M/g viral protein N. Within the limits of experimental error both values are in good agreement with those obtained earlier by a different preparation of the same virus [1].

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STUDIES ON SWINE ENTEROVIRUSES

I. ISOLATION AND SEROLOGICAL GROUPING OF STRAINS

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Summary. Faecal specimens were collected from 568 swine of different age groups from 36 herds. From these 152 (26.7 per cent) chloroform-resistant enterovirus strains cytopathic for swine-kidney cell cultures were isolated. Forty-one strains were selected for examination and purified by the plaque method. Of these 36 could be classified into 13 serological types by means of neutralization tests carried out with immune sera prepared in rabbits against each of 20 of the isolates. Five strains were not neutralized by any of these sera. One of the types gave cross reactions with the viruses of the Teschen and Talfan diseases. Out of the 11 foreign strains only SIBALIN's S 180/4, BETTS' T 80, and T 52A and the Riems 52 strain were neutralized by any of the immune sera prepared against the Hungarian strains.

Numerous authors [2, 3, 5, 9, 10, 13, 14, 15, 16, 19, 23, 24, 26, 27, 28, 29, 31, 33, 35] have succeeded in isolating cytopathogenic agents from intestinal contents of swine, mainly in swine-kidney monolayers. The viruses were designated ECSO (enteric cytopathogenic swine orphan) or ECPO (enteric cytopathogenic pig orphan) viruses on the analogy of the name ECHO used for the designation of similar viruses of human origin.

ECSO viruses are cytopathogenic in swine-kidney cultures, resistant to ether and chloroform, and able to propagate in the intestinal tract of the swine for a period sufficiently long to induce the appearance of antibodies in the blood serum of the host [7, 17, 22]. The particle size of ECSO viruses is about 35 m μ in diameter. According to this characterization the virus of Teschen disease can also be included in the group of swine enteroviruses [21, 34].

ECSO virus strains were classified into 2 to 9 serological types by different authors [6, 9, 21, 28] on the basis of neutralization tests carried out with type sera prepared in rabbits or guinea pigs.

Very little is known about the aetiological importance of ECSO viruses. Some authors have regarded them as real orphan viruses, *i.e.* nonpathogenic; others succeeded in isolating, from the intestinal contents of the swine, enteroviruses, which caused encephalomyelitis in colostrum-deprived, artificially reared piglets [1, 5, 13, 18, 19, 29, 30, 31]. Attempts to reproduce the illness under similar experimental conditions with enterovirus strains isolated from swine affected with gastroenteritis have failed [20, 27].

Materials and methods

Tissue cultures. Cell suspensions were prepared from the kidneys of pigs aged 6–12 weeks by trypsinization at $+4^{\circ}\text{C}$. In the course of this procedure BODIAN'S method was followed which is based on the technique of DULBECCO and VOGT [12]. The sedimented cells were washed and diluted 1 in 150 nutrient medium. Two ml of the dilution were placed in every tube. As growth medium, HANKS' balanced solution completed by 0.5 per cent lactalbumin hydrolysate and 15 per cent calf serum was used. On the 3rd or 4th days, as the monolayer had developed, as maintenance solution sterilized amniotic fluid of 3–5 months' old bovine foetuses was changed for the growth medium. Cultures were used for virus propagation for two weeks after medium had been changed.

Virus isolation was attempted from rectal swabs. These were suspended 1 in approximately 10 HANKS' solution and kept in frozen state, at least, overnight. After thawing chloroform [22], or 500 units of penicillin and 500 μg streptomycin per ml, were added and the specimen was centrifuged at 6000 r.p.m. for 15 minutes. Tubes were inoculated with 0.1 ml of supernatant each. Subsequently the cultures were incubated at 37°C until completely destroyed. Those showing no cytopathic changes during the first two passages were carried over at least five blind passages. However, virus could never be isolated from such blind lines.

Neutralization tests. Before being examined by the neutralization test, the isolates were purified in two steps with DULBECCO'S plaque technique [11]. To prepare type sera, rabbits were inoculated intravenously 6–8 times at weekly intervals with 5 ml of virus propagated in tissue culture. The rabbits were bled on the 8th day after the last injection. For the neutralization test 1:4 to 1:256 dilutions of serum were mixed with equal volumes of a virus suspension containing 100 TCID₅₀ per 0.1 ml. The mixtures were incubated at 37°C for 30 minutes and inoculated into 2–4 tube cultures each, 0.2 ml per tube. The final reading of the test took place on the 6th day. Parallel with the isolates, strains obtained from abroad were tested.

Results

Virus isolation was attempted from rectal swabs collected from 568 pigs in 36 large farms. Cytopathogenic agents were isolated from 152 animals (26.7 per cent).

The strains have been designated with the initial letter or abbreviated name of the stock. When more than one strain was isolated from the same stock, a Roman numeral, the ordinal of the specimen yielding the strain within the stock, was written after the letter(s). In addition to this, the strains before being examined received an Arab numeral written before the letter(s) to facilitate survey (Table I).

Although the number of examinations is insufficient to draw further conclusions, it may be stated that the virus excretion rate was the highest for the pigs aged 2 to 4 months. Besides, excretors were found among 10-day-old piglets as well as among animals aged several years. Examination for enterovirus of 32 sows and their piglets kept in pig-pens isolated from each other in three state farms has shown that piglets may excrete enterovirus with faeces as soon as the 10th day of age. In 8 out of 32 litters tested excretors were encountered and in these litters 15 of the 24 piglets tested (62.5 per cent) proved to be excretor. The piglets were mingled in pig-runs five weeks after birth; after further two weeks enterovirus excretion could be observed in every litter of the three stocks. Enterovirus was isolated from 49 out of the 84 piglets examined (58.3 per cent).

Table I
Serological grouping of strains tested

Serological type	Strains to which type sera were produced	Strains belonging to the type
1.	5-D _{VIII} , 7-Gy _I Teschen, Talfan	4-D _{VII} , 5-D _{VIII} , 7-Gy _I , 15-S _{VII} , 17-SJ _V , 19-SJ _X , Teschen, Talfan
2.	1-AA _{VI} , 33-TK _I	1-AA _{VI} , 33-TK _I
3.	12-PL	12-PL T80, T52A, S180/4, Riems 52
4.	21-Sz	21-Sz
5.	25-T _{VIII} , 41-VL	23-T _{III} , 25-T _{VII} , 36-TV _{IV} , 41-VL
6.	37-U _{VI} , 38-V _I	3-D _{VI} , 6-D _X , 18-SJ _{IX} , 37-U _{VI} , 38-V _I
7.	2-AK _{III} , 10-L _{II} , 40-Ves	2-AK _{III} , 8-Gy _{VI} , 10-L _{II} , 13-PL _{II} , 22-T _{II} , 40-Ves
8.	9-K _{IV}	9-K _{IV}
9.	11-P _{II}	11-P _{II}
10.	16-S _X	14-S _V , 16-S _X
11.	20-SIM _I	20-SIM _I
12.	26-T _{XII}	26-T _{XII}
13.	27-TH _I	27-TH _I , 29-TH _{III} , 30-TH _V , 31-TH _{VI} , 32-TH _{VII}
Untypable strains		
	own strains	24-T _V , 28-TH _{II} , 34-TK _{III} , 35-TK _{IV} , 39-V _{II}
	foreign strains	V ₁₃ , Sibalin: N ₇ , N ₁₁ , U ₁₀ , V ₄ Riems 12, Riems 19

Immune sera were prepared in rabbits against 20 of the strains isolated during the present study. The strains had been purified by the plaque technique before used for immunization. With these sera chess-board neutralization tests were carried out. For comparison, 11 ECSO virus strains received from abroad as well as one strain each of Teschen-disease and Talfan-disease viruses were tested simultaneously. Rabbit immune sera prepared against these two viruses were also included in the study.

On the basis of cross neutralization tests 36 out of the 41 strains isolated by us could be classified into 13 serological types (Table I). Of these types, one including six strains was neutralized, in addition to the homologous type serum, by each of the anti-Teschen-disease and anti-Talfan-disease sera. Five (24-T_V, 28-TH_{II}, 34-TK_{III}, 35-TK_{IV}, and 39 V_{II}) of the 41 strains were not neutralized by any of the type sera; accordingly these have not been classified.

Out of the 11 strains received from abroad, in accordance with the results of other investigators [32], SIBALIN's S180/4 and BETTS' T80 and T52A proved to be related to each other and to the 12-PL strain isolated by us. The strains Riems 52 proved to belong to the same type. None of the other strains received

from abroad were neutralized by any of the type sera used in the present study. These unrelated strains were SIBALIN's N7, N11, U10, and V4, the Riems 12 and 19 strain, and BETTS' strain V13.

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STUDIES ON SWINE ENTEROVIRUSES

II. MORPHOLOGY OF THE CYTOPATHIC CHANGES PRODUCED IN SWINE-KIDNEY MONOLAYERS

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Summary. The morphology of the cytopathic changes produced in swine-kidney tube cultures by forty-one and 11 strains of swine enteroviruses isolated by the author and received from other countries, respectively, were examined in celloidin-embedded preparations stained by various methods.

Based on the morphology of the cytopathic changes the strains have been classified into two groups. One group of strains consistently produced the type of changes signed type-I; this type was characterized by rounding and aggregation of cells, development of morphologically recognizable cell membrane, rhectic destruction or shrinking of nuclei, and the appearance of light perinuclear area. The second group of strains gave rise to morphological changes signed type-II. These changes were introduced by granular destruction of the cytoplasm and appearance of acidophilic bodies resembling cytoplasmic inclusions, in scattered cells. Subsequently, the cytoplasmic granules resemble irregular protrusions surrounding the pycnotic nucleus.

The cytopathic changes produced by each of the strains were consistent and characteristic of the strain. All strains classified into each of the serological types always produced the same type of cytopathic changes.

In previous reports [12, 13, 14] isolation and serological classification of ECSO (enteric cytopathogenic swine orphan) virus strains were described. One hundred and fifty-two strains were isolated from healthy pigs and pigs showing undefined symptoms of encephalomyelitis and/or enteritis. Forty-one of these strains and 11 strains received from other countries were subject to comparative studies and examined in detail. In the present report an account is given of observations on the morphological picture of the cytopathic changes produced by the same strains in swine-kidney monolayers.

Materials and methods

Virus strains. Cytopathogenicity was examined in the 3rd to 5th passages of the original isolates and in successive passages of pure virus lines obtained from the isolates by DULBECCO's plaque technique.

Histological methods. Cultures were infected with 100 TCID₅₀ of virus. Some of these cultures as well as uninoculated control cultures were fixed and stained 6, 12, 18, 36, 48 and 72 hours thereafter.

In situ fixation, and removal from tube, of the cultures were performed according to the technique of ENDERS and PEEBLES [5], which was somewhat simplified in this laboratory. The simplified technique as follows covers the practical requirements.

The medium of cultures grown in tubes of 16 × 160 mm was poured off, the monolayers were thoroughly washed with a buffered solution to remove debris and, subsequently fixed by a histological technique adequate for the chosen method of staining. BOUIN's and HADENHAIN's fixation techniques were employed most often. The preparations that required no fine

examination were fixed with methanol. The fixed preparations were dehydrated with acetone. Then 3–4 ml of a solution containing 7.5 per cent celloidin in a mixture of equal volumes of ethanol and ether were added to each of the preparations, the tubes were tightly stoppered and rolled to let the solution come in contact with the whole inner surface of the tubes. Subsequently the tubes were laid down to have the solution cover the cultures. After 30 minutes celloidine was poured off, the tubes were held downwards and rolled between the two palms until the smell of ether disappeared, i.e. for 5–10 minutes. In the meantime celloidin formed an uniform layer on the inner surface of the tubes. Then cold tap water was added, and poured off after a few seconds. The celloidin film that had formed on the glass wall was then separated by a long anatomic forceps and taken out of the tube. The part containing the culture was cut out, carefully spread on a microscopic slide, and blotted between filter papers. One should be careful to place on the slide the same surface of the culture that had been attached to the glass wall. To fix the monolayer on slide, 2 to 3 drops of caryophilic oil was spread by means of a glass rod on the preparation and allowed to stand there for, at least, 5 minutes. The oil and, subsequently, the celloidin were removed by dissolving them in 96 per cent alcohol (10 minutes) and in a mixture of equal volumes of alcohol and ether, respectively. These procedures were performed in a series of histological tubes; in the first tubes of the series solvent was changed frequently.

The preparation thus perfected can be stained by any staining methods used in histology. We have employed MAYER's acid-haemalaun-eosin, WEIGERT's iron-haematoxylin-eosin techniques and the MALLORY-FARKAS-GIEMSA, methylgreen-pyronin, and PAS (Schiff reaction) staining methods. The stained preparations were dried, cleared up in a phenol-xylol mixture and covered with cover slip using Canada balsam. The preparations thus obtained contained a whole tube culture each.

Results

Based on the cytopathic changes produced in swine-kidney cell cultures 41 strains isolated by us and 11 strains received from other countries could be classified into two large groups (group I and group II). Accordingly, 19 of our own and eight foreign strains belonged to group I whereas 22 of our own and three foreign strains were classified into group II.

In the uninoculated cultures the polygonal kidney cells having dim contours tightly covered the fields. The cytoplasm of the intact cell was homogeneous, in the oval nucleus 2–4 nucleoli were visible (Fig. 1).

Cytomorphology of the cultures infected with group-I strains. The contours of certain cells become sharper and granular degeneration of the cytoplasm appears as soon as 6 hours after inoculation (Fig. 2). At 12 hours some of the cells that have shown initial cytopathic changes appear to be rounded and having hyperchromatic or pycnotic nuclei. The nucleoli are at the beginning well distinguishable; later a light area is seen around each of them. In the cells infected by some of the strains striking karyorrhexis can be observed; the nuclei fall into gross, well-stained granules (Fig. 3). The pyknotic nuclei are surrounded by acidophilic, dense, granular cytoplasm. Certain strains produce a light area of variable width between the morphologically visible cell membrane and the cytoplasm (Fig. 4). This process leads to complete shrinking of the epithelial cells attacked by virus within one or two hours; at the same time no, or hardly any, pathological changes can be detected in neighbouring cells. In general, the cytopathological changes spread over the whole culture within 18–24 hours. The degenerated cells being connected with each other by



Fig. 1. Swine-kidney cell culture on the fourth day of cultivation. Haematoxylin-eosin stain (H—E), $\times 320$

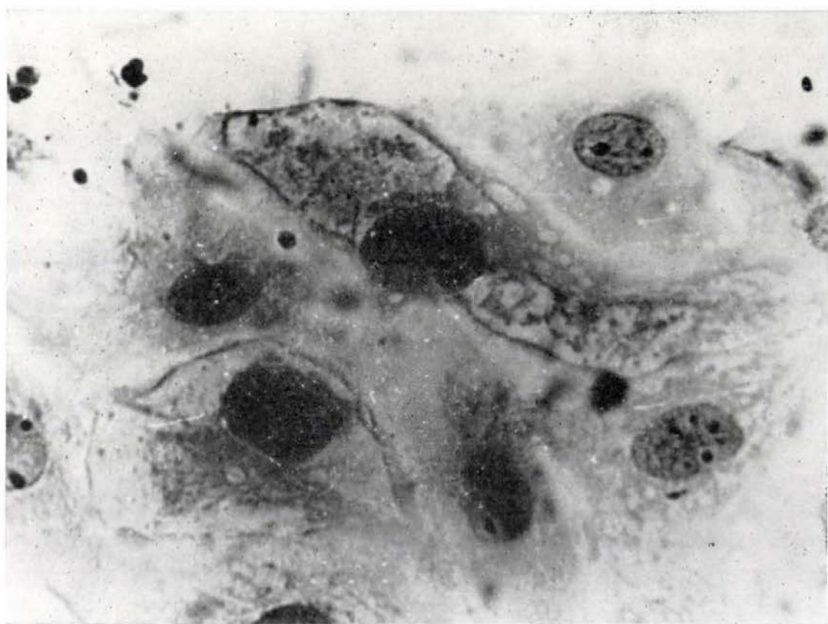


Fig. 2. Type-I cytopathic change. Granular destruction of cytoplasm, sharp cell margins. H—E stain, $\times 800$



Fig. 3. Type-I cytopathic change. Karyorrhexic changes in rounded cells. H—E stain, $\times 1500$

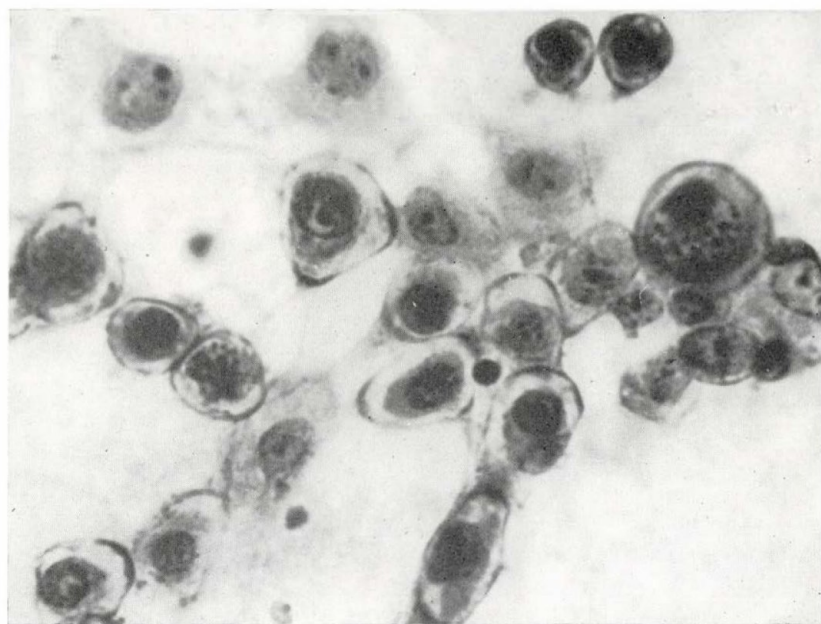


Fig. 4. Type-I cytopathic changes. Perinuclear light area in rounded cells. H—E stain, $\times 800$



Fig. 5. Type-I cytopathic change. Network of rounded cells. H—E stain, $\times 320$

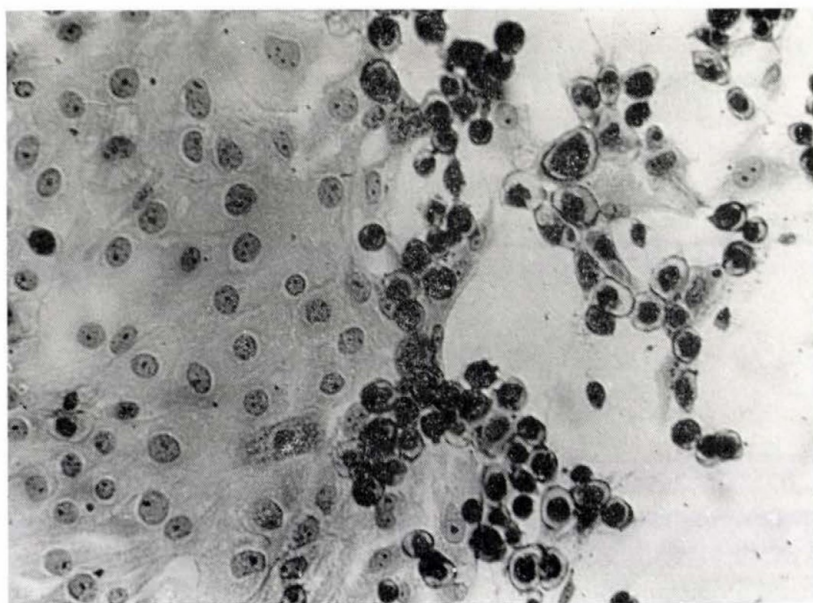


Fig. 6. Type-I cytopathic change. Sharp demarcation of intact and degenerated tissues. H—E stain, $\times 320$

thin filaments show a reticular structure (Fig. 5), or aggregate in groups resembling a bunch of grapes. These groups of cells are sharply demarcated from the intact part of monolayer (Fig. 6). The described cytopathic changes develop very rapidly. Depending on the strain, complete degeneration and detachment from the glass of all cells take 24—36 and 48—56 hours, respectively.

Strain differences were observed in the degree of karyorrhexis, in the width of the perinuclear area, in the occurrence of cytoplasmic vacuoles, *etc.* These differences were characteristic of the strain. The speed of the evolution of cytopathic changes was also variable in the individual strains. Nonetheless, classification of a strain into group-I never caused difficulty even when only living cultures were examined.

Cytomorphology of the cultures infected with group-II strains. This cytopathological picture of explosive character was strikingly different from the one described above.

The initial changes, *viz.* acidophilic, Schiff-positive granules in the cytoplasm of the attacked cell, appear 18—24 hours after inoculation (Fig. 7). In certain cells these granules resemble acidophilic inclusions (Fig. 8). At the same time degenerative phenomena are visible in the nucleus as well.

The nucleus shows pycnotic changes. In cells attacked by some of the group-II strains the nuclear membrane and the nucleoli show more intensive colouration and the former appears to be thickened even before pycnosis is observable (Fig. 9). As degenerative changes progress, the granular remnants of the cytoplasm aggregate around the pycnotic nucleus forming protrusions of irregular margin in the periphery of the cell, in sharp contrast to the rounded cells characteristic of the cultures infected by group-I strains. The group-II-infected cells look like exploded (Fig. 10). Another difference is that initially only scattered cells show pathological changes and these cells do not tend to aggregate (Fig. 11); furthermore, the cytoplasmic granules surrounding nucleus are easily recognizable, even in unstained preparations.

Group-II strains propagate slower than group-I strains in tissue cultures; some group-II strains do not destroy the culture completely; certain groups of cells remain unaffected even for a week after inoculation.

It was a common characteristic of the strains tested so far, irrespective of type of degeneration, that the infective titre rose parallelly with the progress of cytopathic changes and the highest (10^4 — 10^8 TCID₅₀ per ml, depending on strain) were attained simultaneously with the appearance of the most severe changes.

The pattern of pathomorphology proved to be a stable and characteristic marker of the ECSO virus strains under study; neither successive passages nor storage in the refrigerator, or in frozen state, changed this marker. The features that showed some change during passages were the period of time needed for the appearance of the first cytopathic phenomena and that needed for com-

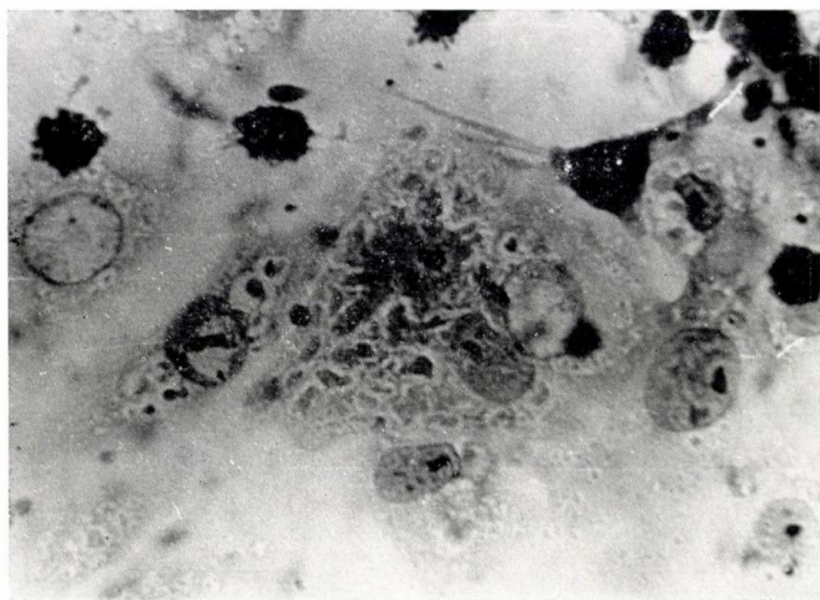


Fig. 7. Type-II cytopathic change. Schiff-positive granules in the cytoplasm, deformed nuclei. Schiff stain, $\times 800$



Fig. 8. Type-II cytopathic change. Gross granules in the cytoplasm causing deformation of nucleus. Schiff stain, $\times 800$

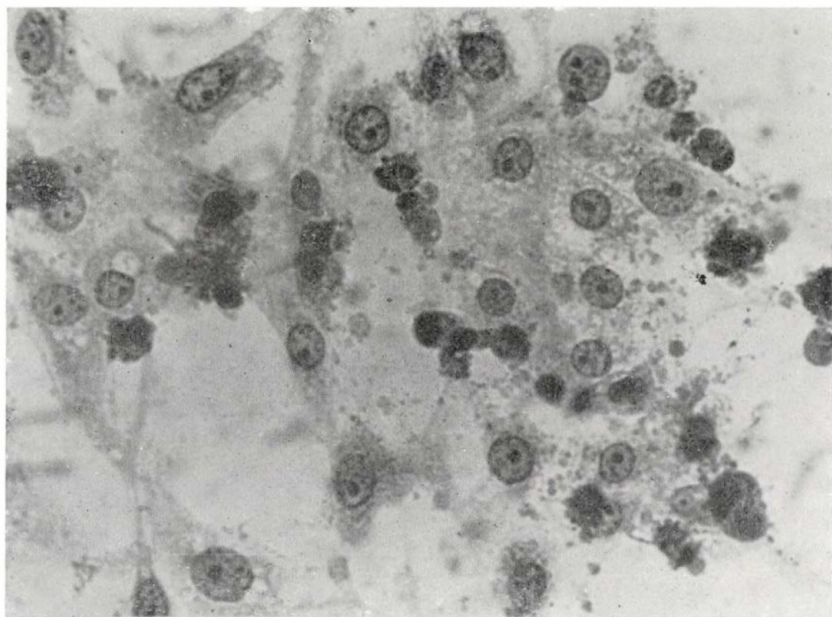


Fig. 9. Type-II cytopathic change. At the initial stage of degeneration the nuclear membrane is prominent and thickened. H—E stain, $\times 320$



Fig. 10. Type-II cytopathic change. Remnants of cytoplasm aggregate in fat-drop-like granules around the pycnotic nucleus. Normal nuclei are also seen. H—E stain, $\times 800$

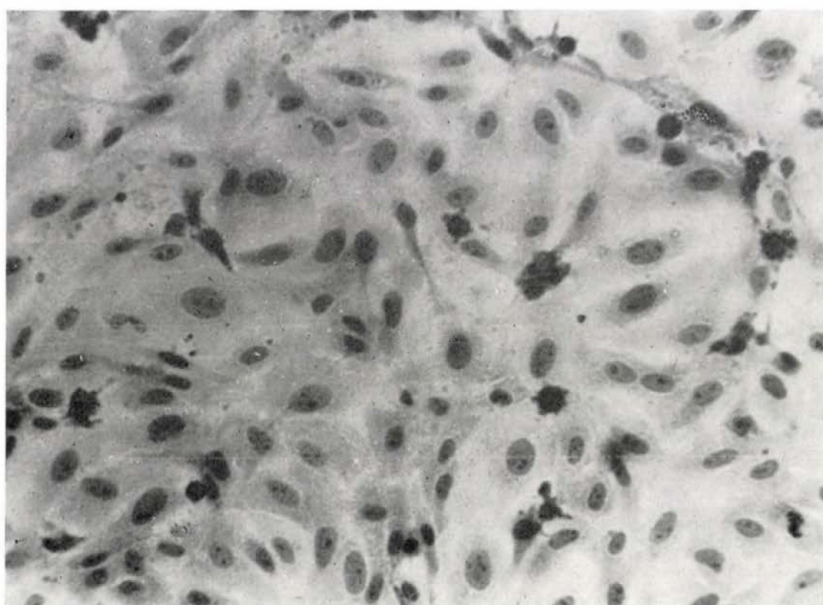


Fig. 11. Type-II cytopathic change. Scattered degenerated cells in the monolayer.
H—E stain, $\times 320$

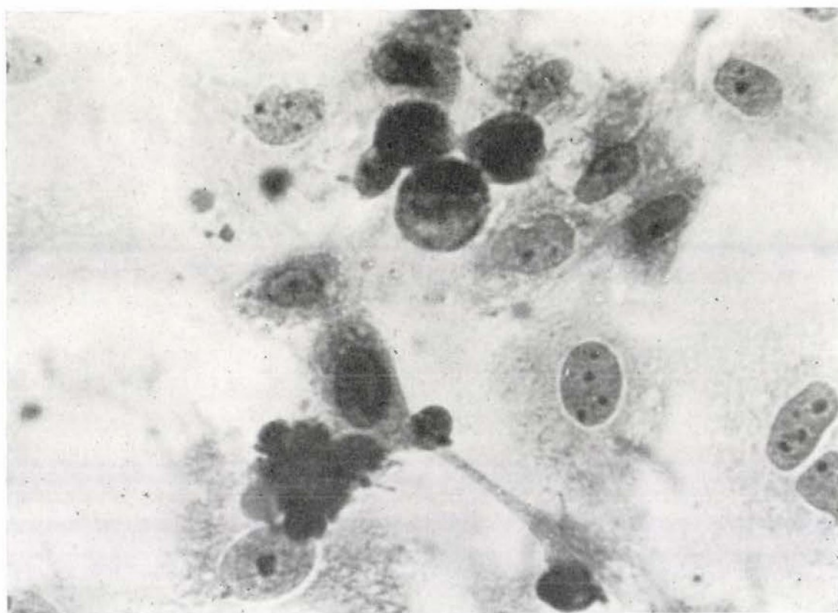


Fig. 12. Type-I (above) and type-II (below) cytopathic changes in a cell culture infected with a mixed strain of enterovirus. H—E stain, $\times 800$

plete destruction of cultures. Both periods were shortening in the course of the first passages.

Two of the 41 of our own strains tested produced the characteristics of both types of degeneration (Fig. 12); in the first three passages of strain 38-V_I beside numerous cells showing type-I degeneration, type-II degeneration was observable. During further passages and plaque purification, the latter type of degeneration disappeared completely.

Strain 37-U_{VI} produced mixed morphological changes even after the first plaque isolation. Two further steps were necessary to separate two lines, one producing type-I pattern and another with type-II morphological picture. The serum of the rabbit that had been immunized with the "mixed" 37-U_{VI} strain neutralized both lines whereas that of the rabbit immunized with the group-I line did not neutralize the other line.

Within each of the immunological types characterized by the neutralization test, every strain belonged to the same cytopathic group confirming that the cytopathic marker is characteristic of swine enteroviruses.

Discussion

Out of the 13 serological types described in the preceding report [14] seven (types 1—7) belonged to the cytopathologic group-I whereas six (types 8—13) to group-II.

Several authors [2, 3, 6] have studied the cytopathic changes produced by porcine enteroviruses in swine-kidney cell cultures. However, most of these authors examined as few as one or two strains each. JENNINGS and KELLY [8] described in detail the cytopathic changes caused by the strain T 80, originally isolated by BETTS [3]. These changes were identical with those characterized as type-I degeneration by us. The T 80 strain as well as BETTS' strain T52A, which is related to T 80, produced type-I degeneration also in the present studies. In addition, type-I degeneration was induced by the foreign strains, Riems 12, 39 and 52 (received from Riems island) and 180/4, N11 and V4 (received from Dr. SIBALIN Stockholm) [16].

We have found a description of type-II degeneration in connection with porcine enteroviruses in two reports. LAMONT and BETTS [9] used the expression "nodular protrusion" for the characterization of the degenerative forms observed in the cultures of their strain designated V13. Our own studies have confirmed that this strain gives rise to type-II degeneration.

Identification of the formations described by JASTRZEBSKI [7] in connection with the two different types described above is more problematic. Based on the published photos and drawings we assume that the "edelweiss" and "network configuration" correspond to type-I degeneration whereas the "crown", "star", "satellite", and "nodular" patterns fit into the type-II

picture. It should be noted that changes reminding of type-II pattern have been described by DUNNEBACKE [4], SHAVER *et al.* [15], and PAY [11] in human amnion cell cultures infected by human poliovirus, in monkey-kidney cell cultures infected by ECHO viruses, and in bovine tongue epithelial cultures infected by the foot-and-mouth-disease virus. Thus, this pathological picture does not seem to be exclusively characteristic of porcine enteroviruses. Our own experiments have shown that certain bovine enterovirus strains may produce type-I-like changes in calf-kidney cell cultures. Moreover, the cytopathic changes produced by each of two foreign and four of our own strains of the Teschen disease virus were indistinguishable from the type-I changes brought about by certain ECSO viruses.

The observations concerning strains 37-U_{VI} and 38-V_I have proved that the intestinal tract of the same pig may be infected by more than one serotype of ECSO viruses. Consequently, reliable typing of these viruses needs purification by the plaque technique.

The conclusions drawn from the morphological picture produced by ECSO viruses may simplify the serological typing of these viruses. It is unnecessary to examine isolates with antisera produced against strains belonging to the other cytopathic group.

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TAXONOMIC STUDIES ON *PROCANDIDA ALBICANS*

I. FERMENTATION OF SUGARS

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Summary. The fermentation reaction in 6 different sugars of 200 *Procandida albicans* (syn. *Candida albicans*) strains has been examined. Glucose and maltose were rapidly fermented by all strains, while lactose and raffinose were not attacked. Galactose and sucrose fermentation differed from strain to strain, and the reaction of the same strain often varied in parallel or repeated examinations. During the 30 day incubation period 84 and 75 per cent of the strains fermented galactose and sucrose, respectively. As the standard identification methods always yield the indicated results, for a more objective characterization of *Procandida albicans* the fermentation pattern symbol dgsm is recommended, where the letters stand for the fermented sugars (glucose, galactose, sucrose and maltose), underlining of a letter indicates the stability of the corresponding property.

In their monograph LODDER and KREGER-VAN RIJ [2] described the fermentation reactions of *Candida albicans* (in our system *Procandida albicans* [6]) as follows. Glucose +, galactose + (often weakly), sucrose — (occasionally some gas bubbles may be produced), maltose +, lactose —. Erroneously assuming that only sucrose-splitting strains are fermenting raffinose, the mentioned authors did not examine the reaction against this sugar. More recently, several species fermenting raffinose but not sucrose have been described. In such strains the action on raffinose is associated with the fermentation of galactose, which is liberated after the hydrolysis of the former [7, 8].

Since the characterization by LODDER and KREGER-VAN RIJ is rather indefinite, allowing considerable error and being even contradictory to the authors' rigid classification differentiating other species by one single fermentation reaction, the examination of the problem from different angles has been thought advisable.

Materials and methods

For the experiments 200 *Procandida albicans* strains were used. The strains had been isolated from patients and were maintained in the culture collection of the Laboratory of Mycology of State Institute of Hygiene. At isolation the strains were identified according to LODDER and KREGER-VAN RIJ [2] and ZSOLT and NOVÁK [11] and classified as *P. albicans* on the basis of their chlamydospore formation, positive sucrose assimilation and mouse pathogenicity [5]. Prior to the examinations the cultures were streaked twice subsequently on the TTC agar of PAGANO, LEVINE and TREJO [9]. The subcultures were finally checked by examining their chlamydospore formation [5].

Basic fermentation experiments were performed in 0.5 per cent peptone water containing various sugars at 4 per cent concentration. The media were distributed at 3 ml amounts into

Widal tubes and sterilized for 30 minutes by steam without pressure [11]. After sterilization the media were checked chromatographically for oligosaccharide decomposition [3]. Prior to inoculation 0.05 ml yeast extract [2] was aseptically added to each tube [11]. The tubes were inoculated with 0.05 ml suspension containing approximately 5×10^8 cells per ml; thus the cell count in each tube was $0.5-1.0 \times 10^7$ per ml. The inoculum was prepared by suspending 48 hour CSILLAG's molasses agar [1] cultures in distilled water. After seeding the tubes were sealed by pouring a layer of sterile mixture of liquid paraffin and petroleum jelly (1 : 1) over the medium. Incubation lasted for 30 days at 26°C .

Departures from the basic methods are described in the text.

Results

The examined 200 strains uniformly failed to ferment lactose and raffinose. Glucose and maltose were rapidly and uniformly fermented by all strains.

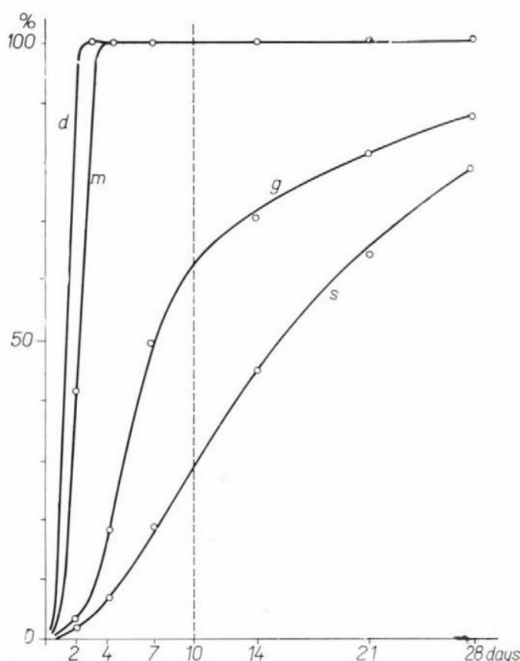


Fig. 1. Period between inoculation and beginning of glucose, maltose, galactose and sucrose fermentation by 200 *Procandida albicans* strains. Results are expressed in percents

The reaction in galactose and sucrose varied from strain to strain. The percentage distribution of 200 cultures according to the period between seeding and the appearance of glucose, galactose, sucrose and maltose fermentation is presented in Fig. 1.

From Fig. 1 it is seen that although some strains fermented galactose and sucrose rapidly, the majority of cultures attacked these sugars after a more or less prolonged incubation. Some strains were negative after the 30 day observation period.

Table I

Distribution of different fermentation patterns according to the beginning of fermentation

Fermentation pattern	Days		
	10	20	30
dm	64	34	4
dgm	90	84	46
dsm	22	24	28
dgs m	24	58	122

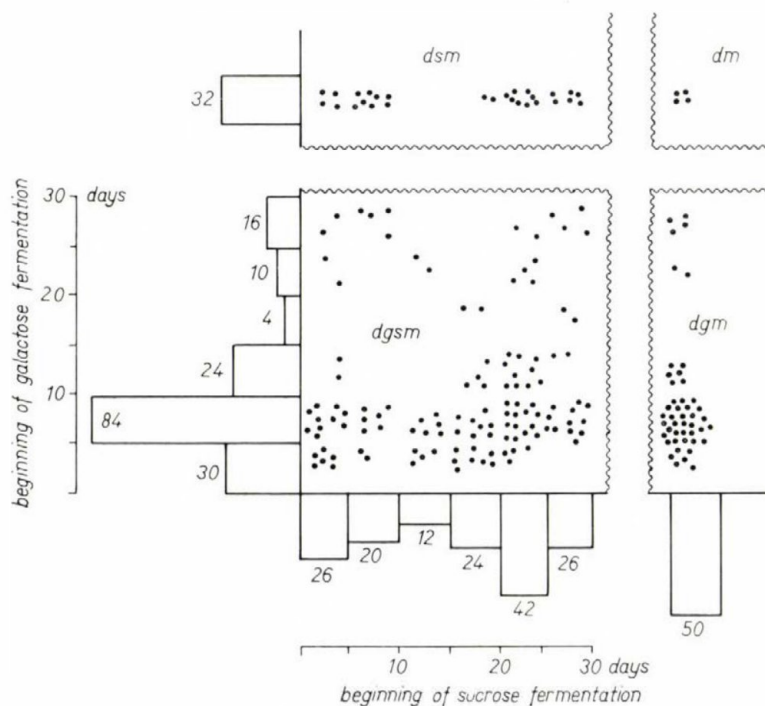
Fig. 2. Distribution of 200 *P. albicans* strains according to the beginning of galactose and sucrose fermentation

Fig. 2 shows the combined distribution of cultures according to the beginning of galactose and sucrose fermentation. The diagram indicates also the four different fermentation patterns observed (dm*, dgm, dsm, dgs m).

The frequency distribution of fermentation patterns obtained after 10, 20 and 30 days of incubation, is presented in Table I.

Because tabulated data may be somewhat misleading (e.g. occurring approximately with the same frequency after various incubation periods, "dsm")

* d = glucose, g = galactose, s = sucrose, m = maltose [7].

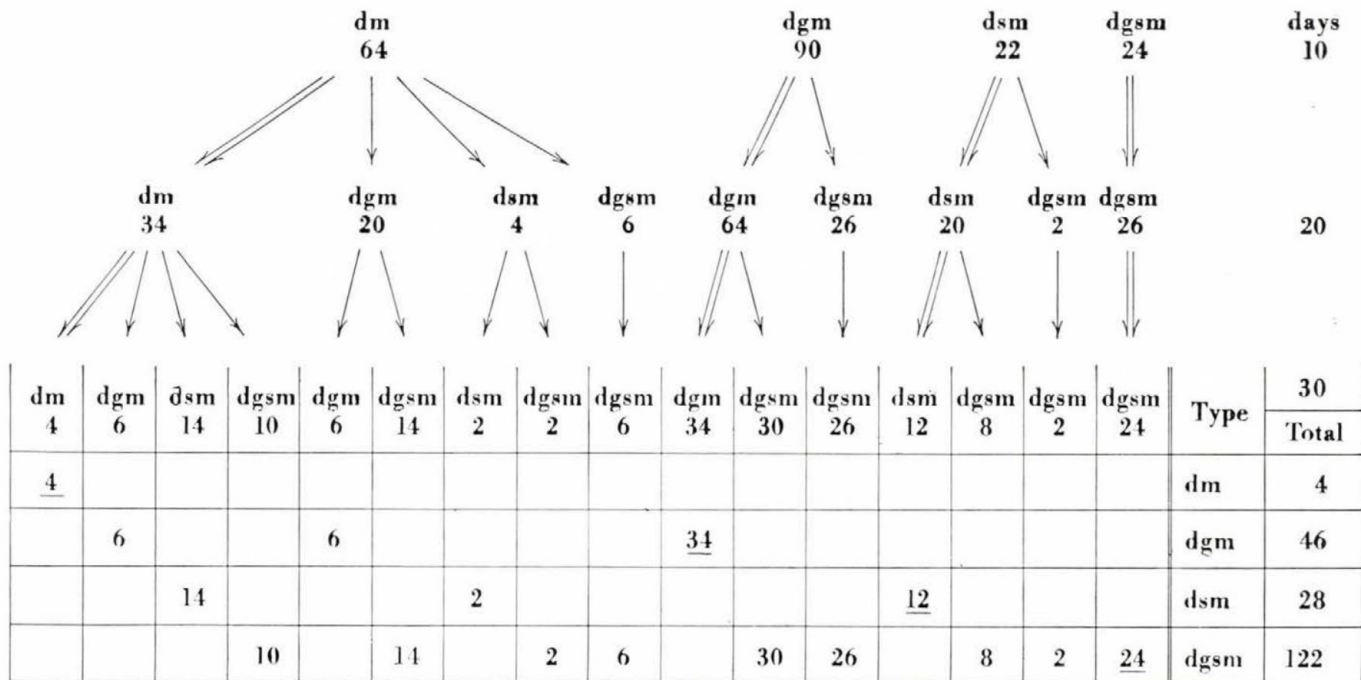


Fig. 3. Further development of 10 day fermentation reactions of 200 *P. albicans* strains. Figures mean the number of strains. Thick arrows indicate stable patterns. Underlined figures mean the number of strains with stable pattern

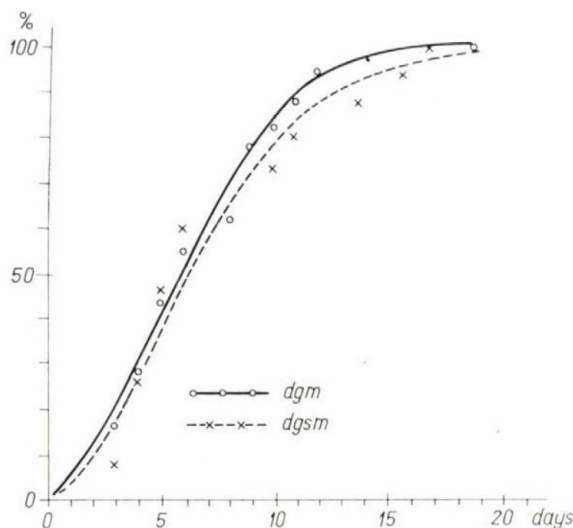


Fig. 4. Beginning of galactose fermentation by pattern dgm and dgsm strains

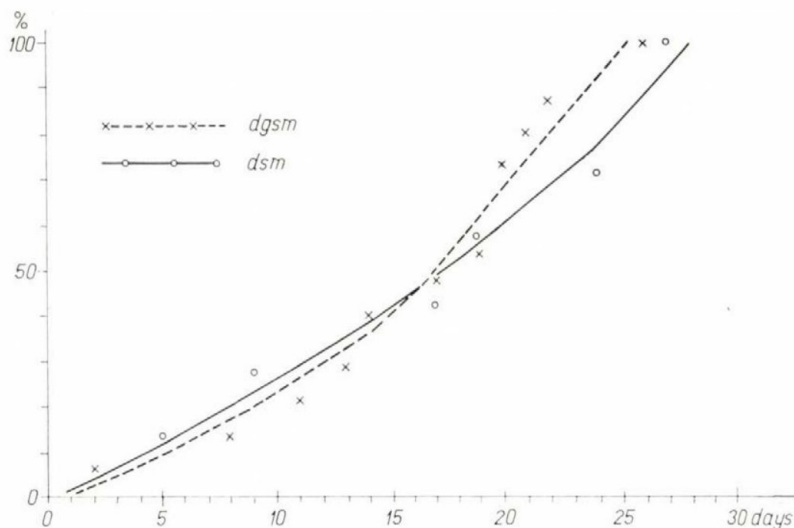


Fig. 5. Beginning of sucrose fermentation by pattern dsm and dgsm strains

character seems stable), in Fig. 3 the development of the fermentation reactions is detailed.

From Table I and Fig. 3 it may be concluded that the fermentation patterns vary considerably with different incubation periods. This was confirmed by the repeated examination of all strains, when the percentage distribution of fermentation patterns was similar. However, the majority of strains gave reactions different from those obtained with the same culture in the previous

experiment. Identical behaviour in the two experiments was observed only with 35 per cent of the cultures.

The association between galactose and sucrose fermentation, in other words the parallelism of the two similarly prolonged reactions, was also examined.

Therefore the appearance of galactose fermentation by dgm and dgsm, and that of sucrose fermentation by dsm and dgsm strains was compared (Figs. 4 and 5).

From the curves presented in Fig. 4 and 5 it is evident that the time of appearance of the two reactions was different. Moreover, the presence (dgsm) or absence (dgm) of sucrose fermentation was not associated with the fermentation of galactose (Fig. 4) and *vice versa*, the presence (dgsm) or absence (dsm) of galactose fermentation was not connected with the fermentation of sucrose (Fig. 5).

Accordingly, as the fermentation of galactose and sucrose develops independently, the existence of two different mechanisms is more likely than an adaptation to the general environment.

It should be noted that parallel tubes generally differed as to the beginning of fermentation. In subsequent experiments 100 parallel tubes were inoculated with the same strain. As expected, the result was almost identical with that shown in Fig. 1.

Although the instability of reactions excluded the possibility of adaptation, the problem was examined in a new series of experiments. The examined strain was inoculated into 10 galactose and 10 sucrose tubes. After incubation, from one of the galactose positive and one of the galactose negative tubes two series of galactose media each consisting of 10 tubes were inoculated. From the sucrose series transfers were similarly performed into sucrose tubes. As a viability control, the corresponding cultures were inoculated into glucose. The results are shown in Figs. 6 and 7.

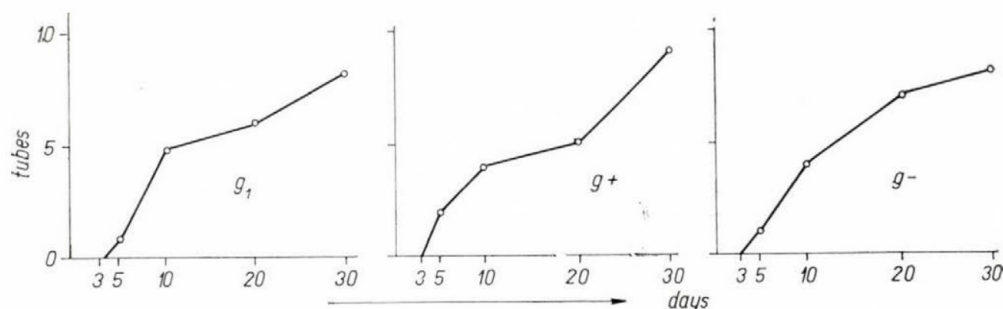


Fig. 6. Stability of galactose fermentation by *P. albicans*. g_1 = Beginning of galactose fermentation in 10 parallel tubes seeded with one *P. albicans* strain. g^+ = beginning of galactose fermentation in 10 parallel tubes seeded with one galactose positive tube of g_1 series. g^- = beginning of galactose fermentation in 10 parallel tubes seeded with one galactose negative tube of g_1 series

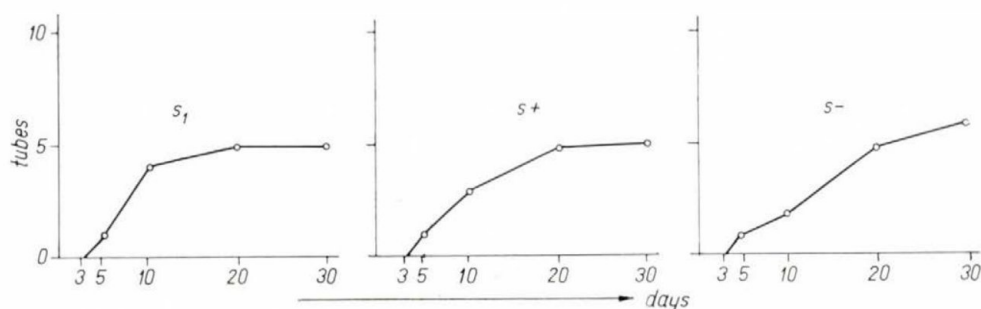


Fig. 7. Stability of sucrose fermentation by *P. albicans*. s_1 = Beginning of sucrose fermentation in 10 parallel tubes seeded with one *P. albicans* strain. s_+ = beginning of sucrose positive tube of s_1 series. s_- = beginning of sucrose fermentation in 10 parallel tubes seeded with one sucrose negative tube of s_1 series

It is clear that, regardless whether the transfer had been made from the fermenting or non-fermenting culture, the results were uniform both in the original culture and in the two kinds of subcultures.

As these experiments excluded the possibility of adaptation only, the problem was studied in further experiments.

First, the inoculum was changed. However, neither a 100-fold increase, nor a 10-fold decrease altered significantly the result or the shape of the curve presented in Fig. 1; only a slight shift to the right or left occurred.

Changing the sugar concentration to 1, 2 and 10 per cent did not cause any important alteration, either; tubes with negative reaction were observed at any of the above concentrations.

As anaerobic conditions might be unfavourable for sugar uptake [10], the fermentation system was preincubated for 24 hours in laid-down tubes with a bent end. It was assumed that by promoting permeation, the oxygen absorbed in the medium might be responsible for the irregular fermentation reactions. Instead of the expected uniformly positive result, apart from a somewhat earlier appearance of fermentation, no significant change was recorded. Negative tubes occurred also in these experiments.

Discussion

The taxonomic signification of our findings may be outlined as follows.

(1) As to fermentation reactions, strains identified by other properties as *P. albicans* are uniform only in glucose and maltose positivity and lactose and raffinose negativity.

(2) As determined by 10 day incubation, as recommended by LODDER and KREGER-VAN RIJ [2], the characterization of *Procandida* (*Candida*) *albicans* was confusing. After 10 days 57 per cent of our strains fermented galactose and 23 per cent sucrose. After 30 days the respective percentages were 84 and 75 (Fig. 1 and Table I). When the results were expressed

in fermentation patterns, after 10 days dgm strains occurred in the highest percentage (45); after 30 days, however, dgsm strains comprised the majority. Considering moreover that incubation for 10 days, apart from the relatively short time needed, is not justified either theoretically or practically, it should be realized that LODDER and KREGER-VAN RIJ's characterization may give rise to misinterpretations.

(3) In repeated or even parallel experiments with the same strain, the fermentation of galactose and sucrose was not uniform and showed a distribution practically similar to that of the summarized results of different strains.

(4) The irregularity of galactose and sucrose fermentation was due not to experimental error. With the worldwide accepted standard methods these anomalies probably cannot be eliminated.

(5) From the experimental results and the above considerations it follows that the sugar reactions of *P. albicans* should be expressed more adequately. The pattern dgsm is recommended with the indication that the fermentation of galactose and sucrose may be sometimes weak or lacking.

(6) Accordingly, the differential diagnostic importance of the stable properties should be emphasized by underlining the corresponding letter. Thus the fermentation pattern for *P. albicans* is dgsm.

The present work indicates that in yeasts with fermenting ability the fermentation of every assimilated sugar or its development by an adaptation-like process is possible. This consideration is supported by observations concerning mainly the maltose fermentation by *Candida solani* [4] and the galactose fermentation by other yeasts. Adaptation-like fermentation by yeasts does not appear with unconditional consistency for every assimilated sugar. This may be due to the different mechanism of anaerobic and aerobic sugar uptake.

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TAXONOMIC STUDIES ON PROCANDIDA ALBICANS

II. DISACCHARIDE-SPLITTING ENZYMES

By

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Summary. It has been observed that 4 *Procandida* (*Candida*) *albicans* strains examined by us split maltose and sucrose intracellularly. As the cells produced no invertase, raffinose was not hydrolysed by living, acetone-treated or homogenized cultures.

It has been concluded that by *Procandida albicans* sucrose and maltose are hydrolysed by different enzymes, and that the enzyme attacking maltose is not identical with yeast maltase, which is capable of splitting also sucrose.

In a previous paper the anomalies observed in the galactose and sucrose fermentation by *Procandida albicans* (syn. *Candida albicans* [8]) have been discussed [12].

It has been revealed that this species is able to ferment sucrose but not raffinose [12]. The purpose of the present study was a detailed examination of the probably invertaseless cleavage of sucrose and its relationship to the hydrolysis of maltose [9–11].

It should be noted that this problem is related to that encountered in the case of *Candida solani*, which likewise ferments sucrose but not raffinose. The explanation of the latter finding has been discussed in a previous study [7].

Materials and methods

Four *P. albicans* strains exhibiting the most stable biochemical behaviour in the previous study [12] were chosen. The fermentation patterns of the strains were, dm* (OKI 360/1960), dgm (OKI 66/1960), dsm (OKI 341/1960) and dgsm (OKI 178/1960).

The strains were grown for 48 hours in Roux bottles containing CSILLAG's molasses agar [1]. The growth was then suspended in saline and washed three times by centrifugation. For the experiments living or acetone-treated cells and cell-free extracts prepared by homogenization with quartz sand, were used [4–7].

Paper chromatography was performed as described previously. Like with *Candida solani*, the cleavage of sucrose, maltose and raffinose was also examined [7].

Results

As the behaviour of all the strains was uniform, results are presented for only one of the cultures.

Living *P. albicans* cells were unable to take up or split sucrose and raffinose during the observation period (Figs. 1 and 2).

* d = glucose, g = galactose, s = sucrose, m = maltose

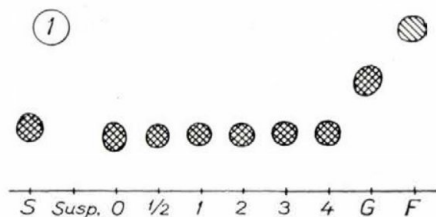


Fig. 1. Sucrose decomposition by living *P. albicans*. M/30, pH 7.2 phosphate buffer, living cells, 700 mg wet weight; 20 mg sucrose in 2 ml volume; 2 μ l aliquot chromatographed. Left, sucrose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

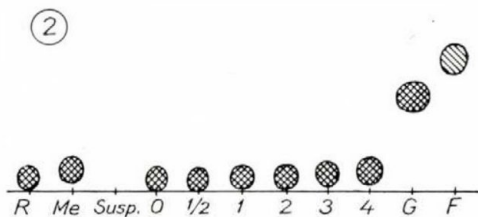


Fig. 2. Raffinose decomposition by living *P. albicans*. M/30, pH 7.2 phosphate buffer, living cells, 700 mg wet weight; 40 mg raffinose in 2 ml volume; 2 μ l aliquot chromatographed. Left, raffinose, melibiose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

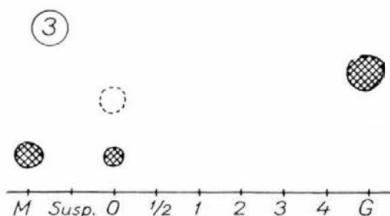


Fig. 3. Maltose decomposition by living *P. albicans*. M/30, pH 7.2 phosphate buffer, living cells, 700 mg wet weight; 20 mg maltose in 2 ml volume; 2 μ l aliquot chromatographed. Left, maltose and suspension controls. Right, glucose control. Numbers on the start line mean the sampling intervals in hours

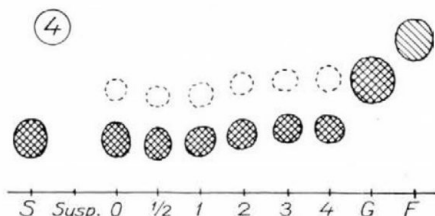


Fig. 4. Sucrose decomposition by acetone-treated *P. albicans*. M/30, pH 7.2 phosphate buffer, acetone-treated cells, 700 mg live wet weight; 20 mg sucrose in 2 ml volume; 2 μ l aliquot chromatographed. Left, sucrose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

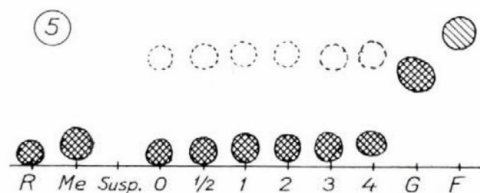


Fig. 5. Raffinose decomposition by acetone-treated *P. albicans*. M/30, pH 7.2 phosphate buffer, acetone-treated cells, 700 mg live wet weight; 40 mg raffinose in 2 ml volume; 2 μ l aliquot chromatographed. Left, raffinose, melibiose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

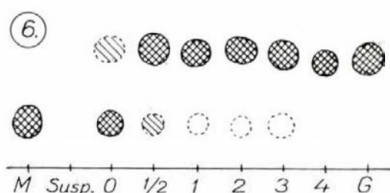


Fig. 6. Maltose decomposition by acetone-treated *P. albicans*. M/30, pH 7.2 phosphate buffer, acetone-treated cells, 700 mg live wet weight; 20 mg maltose in 2 ml volume; 2 μ l aliquot chromatographed. Left, maltose and suspension controls. Right, glucose control. Numbers on the start line mean the sampling intervals in hours

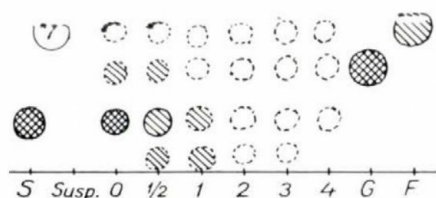


Fig. 7. Sucrose decomposition by the filtrate of homogenized *P. albicans* cells. M/30, pH 7.2 phosphate buffer, filtrate of homogenized cells, 700 mg live wet weight; 20 mg sucrose in 2 ml volume; 2 μ l aliquot chromatographed. Left, sucrose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

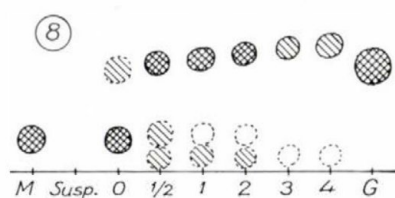


Fig. 8. Maltose decomposition by the filtrate of homogenized *P. albicans* cells. M/30, pH 7.2 phosphate buffer, filtrate of homogenized cells, 700 mg live wet weight; 20 mg maltose in 2 ml volume; 2 μ l aliquot chromatographed. Left, maltose and suspension controls. Right, glucose control. Numbers on the start line mean the sampling intervals in hours

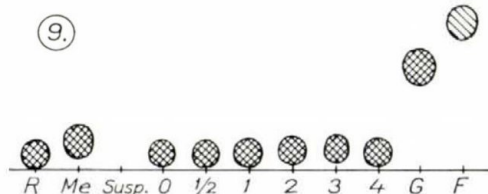


Fig. 9. Raffinose decomposition by the filtrate of homogenized *P. albicans* cells. M/30, pH 7.2 phosphate buffer, filtrate of homogenized cells, 700 mg live wet weight; 40 mg raffinose in 2 ml volume; 2 μ l aliquot chromatographed. Left, raffinose, melibiose and suspension controls. Right, glucose and fructose controls. Numbers on the start line mean the sampling intervals in hours

Maltose was not attacked extracellularly by living *P. albicans* but was taken up into the cells (Fig. 3).

Acetonized cells split neither sucrose nor raffinose (Figs. 4 and 5).

Acetone-treated cells hydrolysed maltose (Fig. 6).

Cell-free filtrates of homogenized cells split both sucrose and maltose (Figs. 7 and 8).

Raffinose was not attacked even by cell-free extracts (Fig. 9).

Discussion

From the results it is evident that *P. albicans* does not produce invertase, because living or treated cells failed to act on raffinose [11]. This conclusion has been confirmed by the lack of raffinose fermentation observed previously [12]. The result at the same time indicates that the raffinose-splitting melibiase, too, is lacking [11].

Accordingly, in *P. albicans*, the cleavage of sucrose (Figs. 1 and 7), as well as that of maltose (Fig. 3, 6 and 8), is intracellular.

The relationship between the cleavage of maltose and sucrose is not clear. It is known that maltase preparations exert also an invertase activity [2, 3, 11]. If maltase were responsible for maltose-splitting, after the elimination of permeation discrimination by acetone-treatment, it would act simultaneously on both sugars [11]. However, as shown in Fig. 4 and 6, similarly to *Candida solani* [7], acetonized cells of *P. albicans* split only maltose. The question now is how acetone exerts its promoting or hindering action on the uptake and decomposition of these sugars.

(1) According to our earlier and present investigations, acetone is undoubtedly capable of bringing about the permeation of sucrose, maltose and even of lactose. Living cells of *Candida pseudotropicalis* take up lactose, but show no extracellular splitting of this substance. After acetone-treatment the cells are possessing a lactase activity, as the hydrolytic products appear extracellularly [5]. The behaviour to maltose of *Saccharomyces carlsbergensis* [5], to sucrose of *Candida solani* [7], and to maltose of *P. albicans*, is analogous. While acetonized *Candida solani* is able to decompose maltose, although its living cells do not take up this sugar [7].

(2) The objection that acetone-treatment does not increase the uptake, and promote only the excretion, of decomposition products, lacks ground, because in acetonized *Candida solani* the decomposition products appeared within a much shorter period than the time elapsed until sugar uptake by living cells had begun [7]. A similar result with maltose was observed in *Saccharomyces carlsbergensis* [5] and in *P. albicans* (present work), and with lactose in *Candida pseudotropicalis* [5]. Thus, it has to be concluded that prior to the excretion of decomposition products the rapid uptake of disaccharides occurred.

(3) According to points [1] and [2], the consideration that acetone-treatment allowed only the re-permeation of the hydrolytic products of maltose, but not those of sucrose, should be discarded, especially because sucrose, maltose and lactose uniformly yield glucose as one of their hydrolytic products.

(4) It would be too far-fetched to assume the complicated condition and complexity of acetone action that would arise if one common enzyme were responsible for the cleavage of both sucrose and maltose. In that case acetone-treatment would make maltose available, and at the same time sucrose unattainable for the same enzyme. With *Candida solani* the untenability of this hypothesis is even more evident, as although both sugars are split intracellularly by that species, at the same time its living cells take up sucrose but not maltose, while the acetonized cells split only maltose, but not sucrose [7]. This example is more relevant than that of *P. albicans*, where the contrast is not so sharp, since living cells of this organism are capable of taking up maltose.

It may be assumed that in *P. albicans* different enzymes are responsible for the cleavage of sucrose and maltose. Moreover, the splitting of maltose

is probably not done by yeast maltase, because in this case the sucrose-splitting activity of maltase would be demonstrable in acetone cells. The elucidation of the problem needs further examinations.

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ISOLATION OF HERPES ZOSTER VIRUS STRAINS

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Summary. Six strains of virus have been isolated from vesicle fluids obtained from eight patients with herpes zoster. The cytopathic lesions observed in unstained human fibroblast cultures, the intranuclear eosinophilic inclusions, the exclusive intracellular localization of the infectious virus, the neutralization tests carried out with paired sera of the patients having yielded virus, and the demonstration of virus antigen in the infected cells with convalescent sera by the immunofluorescent technique, suggested the identity of the strains with the herpes zoster virus.

WELLER [1] was the first to isolate herpes zoster virus. He observed characteristic cytopathic changes in human fibroblastic cultures inoculated with vesicle fluids obtained from patients with herpes zoster. The virus propagated *in vitro* has the peculiarity that infectious particles do not appear in the nutrient fluid, *i.e.* the virus cannot be passaged with the cell-free medium.

Following WELLER's methods we have made attempts to isolate the pathogenic agent from herpes zoster vesicle fluids and to identify the isolates by means of the neutralization test and the immunofluorescent technique.

Materials and methods

Vesicle-fluid samples were taken from eight patients showing the initial stage of herpes zoster by sterile glass capillaries. Human embryonic fibroblast cultures grown in tubes of 16 × 110 mm were inoculated with 0.05 ml of sample each. Depending on the volume of the samples, 2–4 cultures were inoculated with the same material.

Preparation of human fibroblast cultures. Human embryos aged 2–3 months were boned, minced, washed with phosphate buffer solution of pH 7.2, and treated with a 0.25 per cent solution of Difco trypsin overnight. After centrifugation the sediment was re-suspended in the growth medium. Aliquots containing 15 million cells each were distributed in 1-litre Roux flasks. After incubation at 37° C for 10–14 days the cultures were trypsinized and secondary cultures were prepared in tubes of 16 × 110 mm. Three hundred thousand cells in 1.5 ml medium were placed in each of the tubes. The development of monolayers suitable for inoculation required 3–5 days.

The basal component of both the growth and the maintenance fluid was 0.5 per cent lactalbumin hydrolysate in Hanks' balanced solution (70 and 80 per cent, respectively). This was completed by 15 per cent calf serum and 15 per cent bovine amniotic fluid to prepare the growth medium, and with 20 per cent calf serum to prepare the maintenance medium.

Passage of virus. Cell cultures with 14-day-old cytopathic lesions were scraped off the glass wall by a pipette and suspended in 0.8 ml of the maintenance medium. Four cultures of 4–6 days were inoculated with each suspension. The medium was changed on the seventh day after inoculation.

Neutralization tests. Fibroblast cultures were removed from the glass wall by trypsinization (0.25 per cent solution) 8–10 days after inoculation with virus. After centrifugation

the sedimented cells were re-suspended in lactalbumin-hydrolysate-containing Hanks' solution to obtain 2 ml suspension from three cultures. This stock suspension was diluted tenfold and mixed with an equal volume of the serum to be tested. The mixture was incubated at 37° C for 2 hours and diluted with lactalbumin-hydrolysate-containing Hanks' solution to contain 20 per cent serum. Of this, 1 ml was added to each of fibroblast cultures pre-incubated for 2-4 days. The neutralization was expressed in the percentage reduction in the number of foci as compared to the plaque count in the control preparations.

Fluorescent antibody technique. Cover-slip cultures of human embryonic fibroblasts were inoculated with a suspension of cells containing herpes zoster virus. Three days later the cultures were fixed in acetone and overlaid with 1:10-diluted serum. In this state the cultures were kept in a wet box for 30 minutes, washed with phosphate buffered saline (pH 7.4) and treated with fluorescein isothiocyanate-labelled anti-human-horse-gammaglobulin solution for 30 minutes. After another washing and covering with buffered glycerol (pH 7.0), the preparation was examined under the fluorescence microscope.

Stained preparations. Fibroblast cultures were prepared on coverslips of 18 × 18 mm placed in flasks having a basic area of 8 cm². The monolayers thus obtained were inoculated with herpes zoster virus, incubated for three additional days, and stained with haematoxylin and eosin.

Results

Isolation of virus strains and study of their cytopathic characteristics. Cytopathic agents were isolated from six out of the eight vesicle fluids tested (Table I).

Out of the eruptions at different stages of development the vesicles appearing to be the freshest were chosen to yield vesicle fluid. Owing to this principle we succeeded in isolating virus from fresh vesicles as late as the seventh day after the first eruptions had appeared. One of the two failures was due to spontaneous degeneration of the inoculated cultures. In the other negative case the vesicle fluid was heavily contaminated by bacteria.

Table I
Isolation of virus from herpes zoster vesicle fluids

Patient's		Day of taking sample after appearance of first eruption	Virus isolated	Designation of the strain
initials	age, year			
S. S.	56	2.	Yes	Z-1.
I. O.	18	7.	Yes	Z-2.
K. I.	22	3.	Yes	Z-3.
N. Gy.	45	7.	Yes	Z-4.
G. A.	15	2.	Yes	Z-5.
Sz. I.	60	2.	Yes	Z-6.
P. J.	56	3.	No. Spontaneous degeneration	—
F. M.	62	4.	No. Bacterial contamination	—

The early cytopathic changes appeared on the 5th to 8th days after infection. Fig. 1 clearly shows a group of swollen, refractile cells being in sharp contrast with the surrounding intact culture. The process gradually involved the adjacent cells in directions corresponding to the longitudinal axes of the fibroblasts, increase of foci being the more rapid the more compact the culture. Initially only a few foci were visible; the maximum number was observed about the 10th day; no further foci appeared thereafter. On the average, 8—10 foci developed in the cultures inoculated with vesicle fluid.

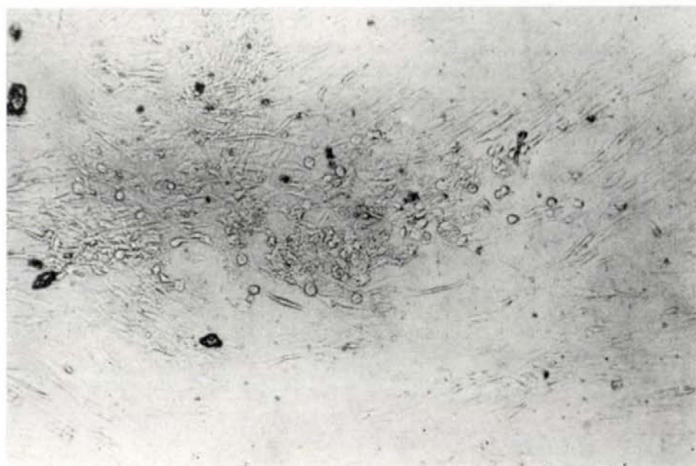


Fig. 1. Cytopathic focus in human fibroblast monolayer two days after its appearance on the 5th day following inoculation with herpes zoster vesicle fluid. Magnification, approx. $\times 300$

As the foci increased, the central cells developed granular degeneration and this was followed by necrosis. In the centres of such foci the monolayer detached itself from the glass (Fig. 2).

Stained preparations show rounded cells of variable size. Aggregation of nuclear granules tend to form an eosinophilic inclusion. The inclusion filling the centre of the nucleus pushes nucleoli and chromatin granules to the nuclear membrane (Fig. 3).

In the progressed stage pycnotic nuclei contain aggregates of small, irregular, rounded, basophilic granules (remnants of chromatin). Among these only residues of inclusions are visible. The final stage of degeneration is characterized by complete destruction of the cells.

Neutralization tests with patients' sera. We wished to prove that the isolates had derived from the vesicle fluids and to identify the isolates with the agent responsible for the clinical picture. For the first purpose paired sera were obtained from four of the patients having yielded virus. From two patients only acute-phase specimens were available. The specimen of one patient proved to be toxic for the fibroblasts.

Table II shows that out of the acute-phase serum specimens those of S. S., K. I., and Sz. I. had very little neutralizing capacity while those of I. O., and N. Gy. neutralized the virus remarkably. The last two were taken after the 5th day of illness. Out of the convalescent samples those of S. S., I. O., and Sz. I. had a well-defined neutralizing capacity, the serum pairs of S. S. and Sz. I. even showed significant rises in this capacity (50-fold and 12-fold, respectively).

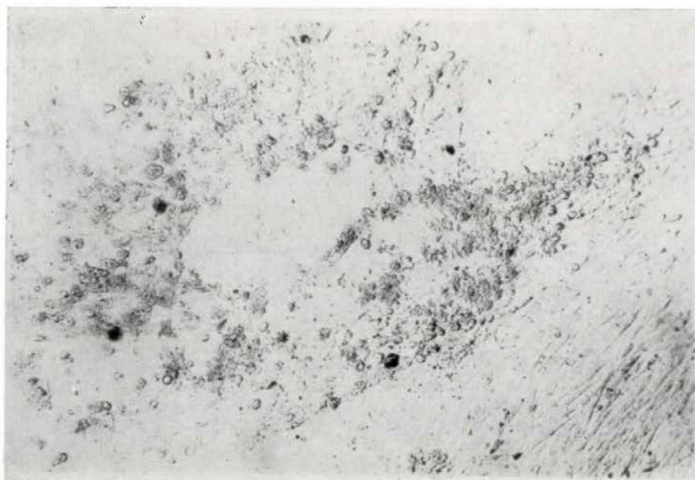


Fig. 2. The same as Fig. 1, six days later. Magnification, approx. $\times 300$

Serological comparison of the isolates. The question whether the strains isolated from different materials were identical with each other also arose. We attempted answer this question in two ways.

Table II
Percentage neutralization by paired sera of patients yielding virus

Initials of patient	Strain isolated from the patient	Day of taking blood sample after onset of illness	Neutralization capacity of serum against own virus
S. S.	Z-1.	2.	10%
		21.	98%
I. O.	Z-2.	7.	92%
		21.	92%
K. I.	Z-3.	3.	10%
N. Gy.	Z-4.	7.	40%
G. A.	Z-5.	cytotoxic sera	
Sz. I.	Z-6.		2.
		21.	95%

* Reduction in cytopathic foci compared with controls where virus was mixed with bovine serum.

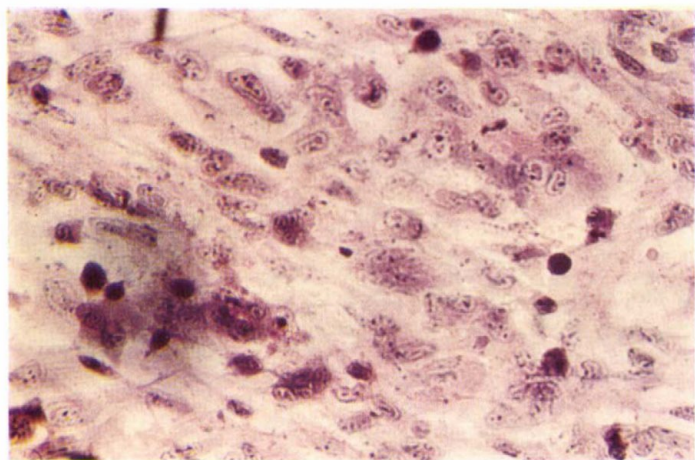


Fig. 3. Type A intranuclear inclusions in fibroblasts infected by herpes zoster virus. Magnification, approx. $\times 1400$

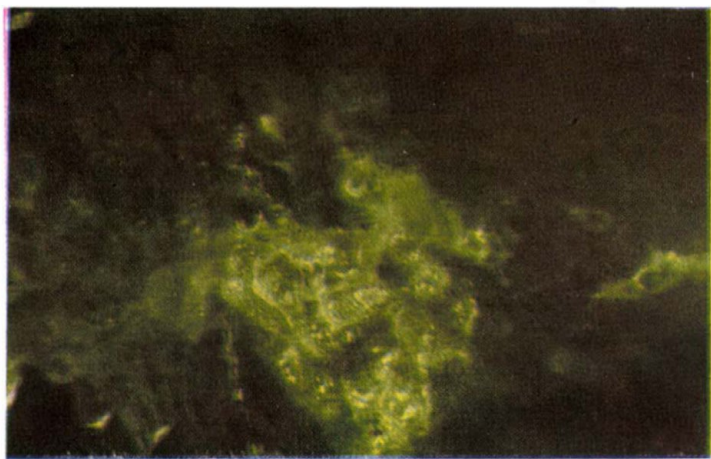


Fig. 4. Specific immunofluorescence in human fibroblasts infected by herpes zoster virus. Z-2 strain, convalescent serum of patient S.S. Magnification, approx. $\times 1000$

(i) We examined whether the serum of each of the patients neutralized the patient's own strain and another strain (Z-1) to the same extent. The paired sera of I. O. (both the acute and the convalescent samples) showed a 4 per cent difference. In the case of Sz. I., the differences amounted to 8 and 5 per cent, respectively, whereas the acute-phase specimen of each of K. I. and N. Gy. neutralized its own strain and the Z-1 strain to the same extent. The results were highly consistent.

(ii) Fixation to six isolates of the antibodies present in the convalescent sample of patient S. S. was examined by the indirect fluorescent antibody technique. The affected cells showed a bright fluorescence regardless of having been infected with the strain isolated from S. S. or with any of the other strains (Fig. 4).

With acute-phase serum samples fluorescence could not be observed whichever of the isolates had been used for inoculation of the cultures.

It can therefore be concluded that neither of the two methods showed any difference between the isolates.

The adequateness of the immunofluorescence test as a diagnostic procedure. Serum pairs of four patients suffering from herpes zoster were tested by the immunofluorescence technique. In two acute-phase specimens no antibody was found while the other two contained considerable amounts of antibody. The antibody concentration was high in each of the four convalescent samples (Table III).

Table III

Antibodies to the Z-1 strain in the sera of four patients with herpes zoster as demonstrated by the fluorescent antibody method

Initials of patient Blood sample	N. S.		L. T.		T. J.		G. D.	
	A.	R.	A.	R.	A.	R.	A.	R.
Degree of fluorescence	—	++++	—	++++	+++	++++	++	+++

A = acute-phase serum

R = convalescent serum

Discussion

The agents isolated by us from herpes-zoster vesicle fluids agreed with those isolated by WELLER [1] (i) in producing focal lesions in human fibroblast cultures; (ii) in the character of development of the lesions; (iii) in the absence of extracellular virus; and (iv) in the contact way of spreading from cell to cell.

In the present study the technique of taking sample was different from that used by WELLER. We sucked the vesicle fluid into a glass capillary and inoculated the undiluted fluid into tissue cultures immediately, *i.e.* there were no coagulation and, consequently, dilution in buffer solution or milk could be

omitted. We attribute the high isolation rate to this modification. Unlike WELLER we did not succeed in either isolating or maintaining herpes zoster virus in HeLa cell cultures.

GOODPASTURE and ANDERSON [3] and, subsequently, BLANK *et al.* [4] inoculated human skin fragments implanted on the chorio-allantoic membrane of the embryonated hen egg with vesicle fluids obtained from patients with herpes zoster. In this manner they produced typical, eosinophilic, intranuclear inclusions. A great number of such inclusions was observed by WELLER and STODDARD [5], who inoculated Maitland-type cultures with varicella vesicle fluids, and by WELLER *et al.* [2], who infected human fibroblastic cover-slip cultures with zoster vesicle fluids. The inclusions observed by us were identical with those described by the cited authors. Such inclusions were demonstrable in the infected cells before the first cytopathic changes had become observable in the unstained cultures.

Several methods have been employed to demonstrate the immune response of patients to herpes zoster virus. NETTER and URBAIN [6] agglutinated with convalescent sera elementary bodies obtained from vesicle fluids; BRAIN [7] and, later, WELLER and WITTON [9] and TAYLOR—ROBINSON [8] used the neutralization test. WELLER and WITTON [9] and TAYLOR—ROBINSON and DOWNIE [10] carried out complement fixation tests with concentrated nutrient fluids as antigen.

In the neutralization test we applied a technique different from those used by WELLER [9] or TAYLOR—ROBINSON [8]. In accordance with the intracellular localization of the virus, neither of these authors, who tested serial dilutions of serum, observed complete neutralization.

The neutralization tests carried out by us have brought evidence that the isolated agents had derived from the patients; cross neutralization tests showed that the isolates were identical with each other.

WELLER and COONS [11] and, later, KOLLER *et al.* [12] pointed out that among the available methods the indirect immunofluorescence test is the most reliable for identification of these pathogenic agents. Using this test we have confirmed that the strains isolated from our six patients were identical with each other and, as far as possible, the responsibility of the agents for the illness was also confirmed. The method may be useful when the aetiological diagnosis of herpes zoster is to be confirmed.

Acknowledgement. The authors are indebted to Mrs. E. Dóczy for valuable technical assistance.

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QUANTITATIVE PRECIPITATION AND ENZYME INHIBITION BY ANTIPHOSPHORYLASE*

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Summary. Quantitative precipitation and enzyme inhibition by homologous rooster antisera prepared against crystalline rabbit muscle phosphorylase *b* have been analysed. It has been shown that when the relative inhibition is under 66 per cent, the inhibiting effect of sera is proportional to the antibody content. The inhibiting action of sera was expressed in antiphosphorylase units. As compared to that neutralized at the equivalence point, in the case of excess of antigen the same amount of antiserum was able to inhibit the activity of a two-fold amount of phosphorylase. The correlation between enzyme inhibition and the precipitating antibody content of the sera was only approximately proportional. The causes of this phenomenon is discussed.

In a previous paper [1] it has been shown that immune sera prepared in the rabbit against rooster muscle phosphorylase exert not only a precipitating but also an enzyme inhibiting effect. In 1958 FISCHER and KREBS elaborated a procedure for the preparation of crystalline rabbit muscle phosphorylase *b* [2]. The new technique was highly superior both in yields and reproducibility to the previously used method [3].

In the present study, by analysing antiphosphorylase sera prepared in roosters, answers were sought to the following question. (i) When is the antibody content proportional to the enzyme inhibiting effect of antiphosphorylase. (ii) What kind of connection exists between the antibody content and inhibiting effect. These questions were considered particularly important, because in the phosphorylase—antiphosphorylase reaction the inhibition of enzyme activity is a sensitive indicator of the specific antibodies present in the system, and it is suitable for the determination of small quantities of antibody. Such investigations may mean a new progress in the field of the microdetermination of antibodies.

Materials and methods

Preparation of phosphorylase b. Rabbit muscle phosphorylase *b* (in the following phosphorylase) was prepared and crystallized three times as described by FISCHER and KREBS [2].

Preparation of antisera. Roosters were immunized at 5 day intervals with gradually increasing doses of phosphorylase as follows. First one intravenous injection of 0.5 mg, then intramuscular injections of 2.0, 8.0, 20.0 and 30.0 mg phosphorylase were given. The animals were bled 5 to 7 days after the last injection. The blood was left to stand for 2 hours at 37° C.

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then for 1 day at 5° C. At intervals the serum was decanted and collected. After centrifugation the sera were stored at 0° C.

Estimation of enzyme activity and inhibition. Phosphorylase activity was assayed as described by ILLINGWORTH and CORI [3], in a total volume of 0.8 ml at pH 6.8 in the presence of 1 mM AMP.* In order to stabilize the enzyme, instead of cysteine, 1 per cent of normal inactivated rooster serum was added to the diluent solution (0.9 per cent NaCl, 2 mM EDTA, 10 mM Tris, pH 6.8). Cysteine had to be omitted, as its excess inhibited the precipitation of phosphorylase, and after prolonged storage it disturbed the reaction by becoming partly insoluble. Diluted with serum or with cysteine, the enzyme showed a uniform degree of activity.

For the titration of antisera, to 0.2 ml suitably diluted phosphorylase preparation 0.2 ml immune or normal serum dilution was added and the system was incubated for 30 minutes at 30° C. After the addition of 0.4 ml substrate (0.034 M glucose-1-phosphate, 2 per cent glycogen, 2 mM AMP, pH 6.8), the tubes were further incubated at 30° C for 10 minutes. The reaction was stopped by 3.2 ml 5 per cent trichloroacetic acid and the determination of *P* was carried out in a 2 ml aliquot of the filtrate as described by TAUSSKY and SHORR [4].

Phosphorylase activity was expressed in arbitrarily chosen units, 1 unit corresponded to the amount of enzyme liberating 1 μ g inorganic *P*, provided that the substrate was decomposed to less than 20 per cent. *E.g.* when under the indicated conditions 0.2 ml of a 1 : 10 diluted phosphorylase preparation liberated 20 μ g *P* (= 20 phosphorylase units), the original undiluted preparation contained 200 units in 0.2 ml or 1000 units in 1 ml.

Quantitative precipitation. Quantitative precipitation was performed with rooster antisera inactivated at 56° C for 30 minutes and centrifuged prior to the experiment. To equal aliquots of the immune serum, increasing amounts of the antigen solution were added. The tubes were incubated at 37° C for 2 hours, then at 4° C for 3 days. The precipitate was washed 3 times in buffered saline by centrifugation at 4° C [5]. The protein content of the precipitate and antigen was determined by means of an Unicam spectrophotometer at 280 μ m wavelength. As protein standards rooster gamma-globulin and crystalline rabbit muscle phosphorylase were employed.

Results

Measurement of antiphosphorylase content of sera. First, the conditions ensuring a proportional correlation between enzyme inhibiting effect and the amount of sera were determined.

As shown by the neutralization curve in Fig. 1, if gradually increasing aliquots of antiserum are added to equal aliquots of phosphorylase, within a certain range the degree of inhibition was proportional to the amount of added serum. At a relative inhibition above 56 per cent the curve showed a break. It is clear that with a considerable antigen excess, the degree of inhibition is directly proportional to the amount of antiserum. The antiphosphorylase content cannot be expressed adequately with the percentage of relative inhibition, it being the function of the enzyme quantity actually used. The antiphosphorylase content of a given serum may be more correctly defined on the basis of its capacity of inhibiting the activity of known units of phosphorylase. As shown in Fig. 2, in this manner the degree of inhibiting effect could be expressed independently of the amount of enzyme.

Fig. 2 presents phosphorylase activity and the inhibiting effect of sera as a function of enzyme concentration. Phosphorylase activity is given in arbitrary units *versus* the amount of phosphorylase in ml. Curve 1 shows that,

* Abbreviations: AMP, adenosine 5-phosphate; EDTA, ethylenediaminetetraacetate; Tris, tris-oxyethyl-aminomethane.

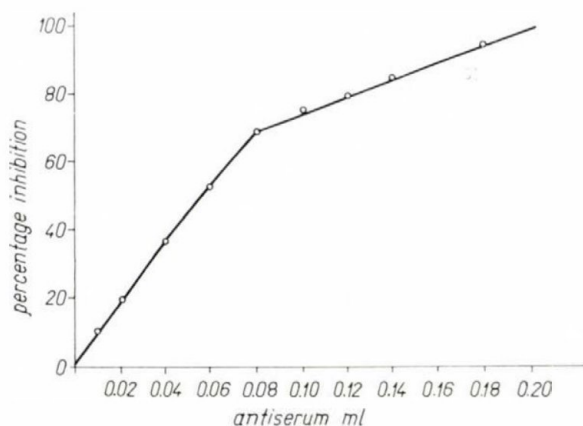


Fig. 1. Relationship between the inhibition of phosphorylase activity and amount of immune serum

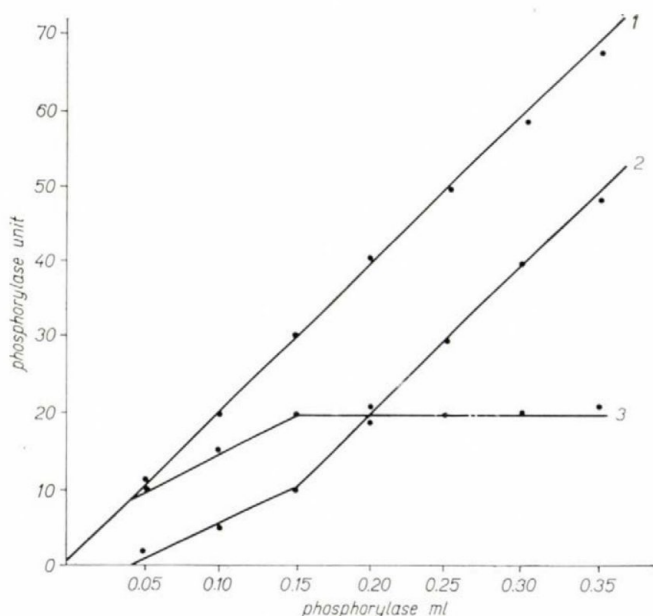


Fig. 2. Relationship between the inhibition of phosphorylase activity and amount of enzyme. Curve 1: 0.04 ml control serum; Curve 2: 0.04 ml antiphosphorylase serum; Curve 3: Inhibition calculated from the difference between curves 1 and 2

in the presence of 0.04 ml control serum, phosphorylase activity is directly proportional to the amount of enzyme. Curve 2 represents the phosphorylase activity estimated in the presence of 0.04 ml immune serum. Curve 3 shows in antiphosphorylase units the inhibiting values calculated from the difference between the two former curves. As seen from the shape of Curve 3, when the enzyme is given at increasing amounts to equal aliquots of immune serum,

inhibition is at first total due to an antibody excess. When the amount of enzyme is further increased, residual activity shows a constant increase, while the percentage inhibition a constant decrease; absolute inhibition values increase gradually until the relative inhibition decreases to 66 per cent. From that point onward, independently of the added amount of enzyme, the absolute inhibition values remain constant. From the curves it follows that for the

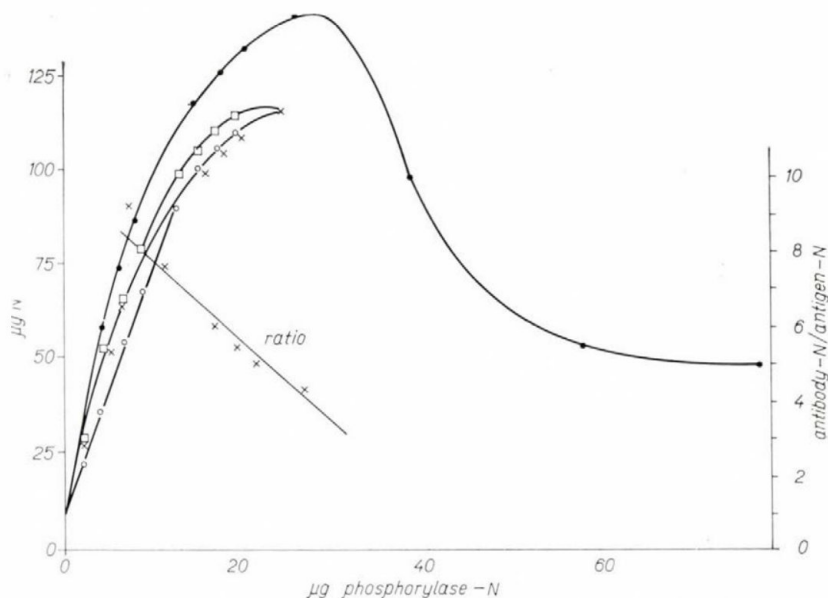


Fig. 3. Quantitative precipitation of phosphorylase. ●—● total precipitate N; ×—× antibody N of the precipitate; ○—○ antibody N in the precipitate calculated from antibody $N = 8.6 \times 0.162x^2$; □—□ antibody N in the precipitate calculated from antibody $N = 14x - 1.83x^3/2$.

measurement of antiphosphorylase activity it is important so to choose the proportion of enzyme, and antibody that the relative inhibition remain under 66 per cent. Especially if dilute enzyme or antibody solutions are used, it is advisable to set this limit at 40 per cent. Under these circumstances the inhibition values expressed in antiphosphorylase units are proportional to the antibody content.

Quantitative precipitation of phosphorylase. In Fig. 3 data of quantitative phosphorylase precipitation are shown. The addition of increasing amounts of antigen results in an increase of the precipitate until the equivalence point is reached. With the aid of supernatant analysis the antibody content of the precipitate could be calculated. In Fig. 3 it is shown that the estimated antibody content can be expressed satisfactorily by both square and fraction exponent

equations. From this test it might be concluded that the system was homogeneous and did not differ in behaviour from other proteins.

Quantitative supernatant analysis. In addition to the conventional precipitation technique, the supernatants of quantitative precipitation systems were analysed for activity. First, the residual phosphorylase activity of supernatant aliquots was determined. To other supernatant aliquots phosphoryl-

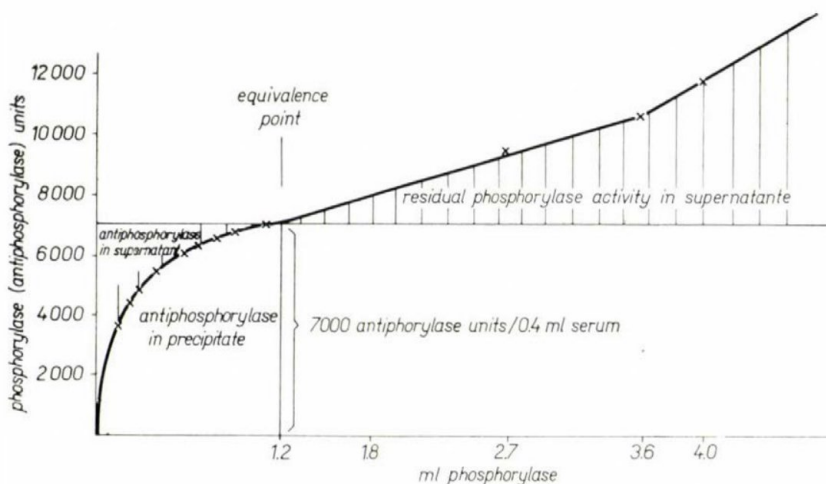


Fig. 4. Quantitative analysis of supernatants

ase of known activity was added, and the measure of inhibition was subsequently determined. Thus the neutralization point, as well as the quantitative relationships between the reactants in the case of antigen, respectively antibody excess, could be exactly estimated. The quantitative supernatant analysis is demonstrated in Fig. 4.

Supernatant analysis performed by assaying the activity is not only a quantitative method but, especially for antibody determination, one more sensitive than the conventional precipitation test. Near to the equivalence point antibodies are difficult to determine by precipitation. In contrast, estimation of the activity, yields a reliable result. Particularly in the case of antigen determination, however, it is advisable to check the result of supernatant analysis by precipitation, because in this way the enzymically inactive antigen-antibody complexes present in the supernatant can also be detected. Besides, it should be realized that the precipitation analysis of the supernatant is needed also for confirming homogeneity.

Correlation between the inhibitory effect and the antibody content of sera. After it had been revealed that in the case of an excess of antigen inhibition was proportional to the antibody content of sera, the correlation between inhibition

and precipitation curves, *i.e.* (1) the situation of the equivalence point on the inhibition curve, (2) the amount of antibody corresponding to 1 antiphosphorylase unit, had to be elucidated. For this purpose the quantitative precipitation of antiphosphorylase sera was performed and the residual phosphorylase activity of the supernatants was measured. Sera of non-immunized roosters served as controls. The results are given in Fig. 5.

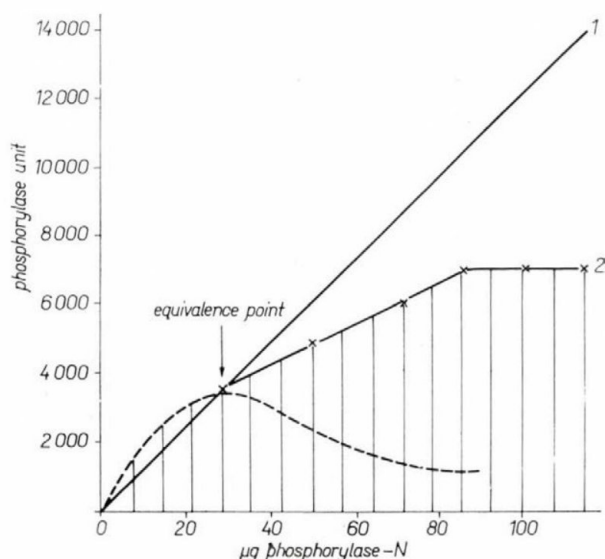


Fig. 5. Inhibition and quantitative precipitation of phosphorylase. Curve 1: Phosphorylase added in units; Curve 2: Phosphorylase inhibited in units; Dotted line: Total precipitate N

The amounts of phosphorylase N are plotted on the abscissa, while the ordinate represent phosphorylase activity, and inhibiting effect of sera. The area between line 1 and the abscissa indicates the activity of added phosphorylase in the absence of immune serum.

The shaded area between line 2 and the abscissa shows the amount of neutralized phosphorylase. The unshaded area between lines 1 and 2 represents the residual phosphorylase activity. The produced precipitate expressed in N values is shown by the dotted line. The equivalence point is also indicated. The shape of the inhibition curve is similar to that displayed in Fig. 2. It is seen that the point of total neutralization coincides with the equivalence point. Until relative inhibition had decreased to 66 per cent, as the amount of enzyme was increased, the residual phosphorylase activity as well as the absolute inhibition values also increased. Beyond that point the absolute inhibition values remained constant, regardless of the amount of enzyme added. As compared to that observed at the equivalence point, in the case antigen excess the same amount of

immune serum was able to inhibit a two-fold amount of phosphorylase. *E.g.* with antigen excess, 0.4 ml serum inhibited 14 000 units of phosphorylase, whereas for the precipitation of all the antibodies present only 7000 units needed. A decline in the inhibition curve occurred when the antigen had reached the threefold of the concentration at the equivalence point. It should be noted that residual phosphorylase activity was not influenced by previously freeing the system from the precipitate.

Two antisera gave inhibition curves different from those obtained with the others. With these sera the neutralization point fell into the zone of antigen excess. Thus in the range between the equivalence and neutralization points, the antigen excess in the supernatant could be shown only by precipitation. From this it follows that the presence of soluble antigen-antibody complexes characteristic of antigen excess, that exert no enzyme activity, should always be considered.

In further experiments the quantitative correlation between the inhibiting effect and the antibody content of sera was examined. By quantitative precipitation the antibody N was estimated, and the inhibition of phosphorylase activity was determined. The results are presented in Table I. The antibody content varied from 63 to 526 μg antibody N/ml, the antiphosphorylase content, from 1848 to 36,852 units. In the last column is shown the amount of antibody N corresponding to 1 antiphosphorylase unit. It is clear that apart from serum 5, the values are fairly uniform and, on the average, 1 antiphosphorylase unit corresponds to 0.016 μg antibody N.

Table I
Antibody content of antiphosphorylase sera

Designation of sera	Antibody N $\mu\text{g/ml}$	Antiphosphorylase units/ml	Antibody N μg per antiphosphorylase units
1	71	4,000	0.017
2	290	17,740	0.016
3	143	7,714	0.018
4	526	36,852	0.014
5	63	1,848	0.033

Phosphorylase-antiphosphorylase union. While in the excess of antibody the ratio of reacting antigen and antibody can be calculated from the N value of the precipitate, in the case of excess antigen a determination of the free antigen should be carried out. Provided that the antigen is of enzymic nature, this can be done in a simple way. The estimation of activity is advantageous also for other reasons. When the inhibition experiment is carried out in the case of

antigen excess, probably only the antibody reaching with the enzymically active centre (substrate-binding group) of phosphorylase is measured. Antibody molecules, blocking enzymically inactive determinant groups of phosphorylase, do not inhibit enzyme activity in antigen excess. Thus, in enzyme inhibition tests a relatively definite group of the antibodies is measured. The results

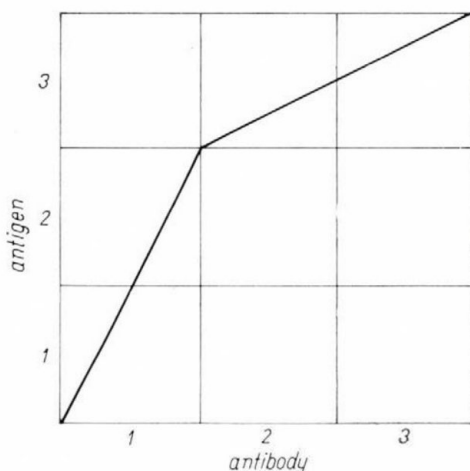


Fig. 6. Corrected inhibition curve versus antibody concentration schematically showing phosphorylase-antiphosphorylase reaction

have shown that with an excess of antigen the antiphosphorylase is able to unite with twice more antigen than that needed for its precipitation at the equivalence point (Fig. 5). It is interesting to compare Figs. 1, 2 and 5. The break in the inhibition curves uniformly occurs at 66 per cent relative inhibition. Fig. 1 shows inhibition in antigen excess as a function of the amount of immune serum. It may be seen that with a large excess of antigen the slope of the curve is twice steeper than at the neighbourhood of the equivalence point. In other words, with much antigen in excess, 1 part of antibody unites with 2 parts of antigen, while near to the neutralization point 1 part of antigen is neutralized by only 2 parts of antibody. Fig. 6, which has been obtained by lengthening the ends of the straight parts of the inhibition curve shown in Fig. 1, expresses these circumstances schematically. The corrected inhibition curve clearly confirms the conclusions drawn from the curve showing inhibition *versus* enzyme concentration.

If with an excess of antigen, at the neutralization point the antigen-antibody proportion is arbitrarily taken as 1, up to 66 per cent relative inhibition 1 part of antibody is used for the neutralization of 2 parts of antigen; *i.e.* the antibody-antigen ratio equals $1/2$. Between 66 and 100 per cent relative

inhibition, for the neutralization of 1 part of antigen 2 parts of antibody are used up, thus here the antibody-antigen ratio corresponds to 2. The composition of antigen-antibody complexes depends on the quantity, avidity and valency of the reacting components.

Experimental data indicate the heterogeneity of antibodies [6]. Therefore it may be expected that different disturbing effects disfigure the clear situation outlined above (1). Antibodies that are active not only against one determinant group may be also inhibitory (2). Antibodies reacting with one determinant group may differ in avidity, valency, *etc.*; the difference may be responsible for a deformation of the ideal shape of the curve. The presented curves were obtained with sera containing the disturbing factors only in minimum amounts. Antiphosphorylase sera giving curves of somewhat different shape were also encountered. These differences were attributable to the mentioned factors and to the heterogeneity of antibodies.

Discussion

When the inhibiting effect of sera is plotted against the amount of enzyme, the antiphosphorylase activity is proportional to the antibody content only when the percentage value of inhibition is under 66. In the practical determination of antiphosphorylase content, this value should be limited to 40 per cent, as the equilibrium is completed only after a prolonged period. For the characterization of the enzyme inhibiting effect of antiphosphorylase sera the result should not be given percentage, because this value varies with the actually used amount of enzyme. It is more advantageous to express the activity in antiphosphorylase units. One unit of antiphosphorylase is the amount of antibody that, under standardized conditions and at 40 per cent or lower relative inhibition, neutralizes the activity of 1 unit of phosphorylase.

The break of the inhibition curves in the case of excess of antigen is due to the fact that, as compared to the union at the equivalence point, with more antigen in excess, antiphosphorylase and phosphorylase are bound in an altered proportion. If at the equivalence point x units of phosphorylase antigen are needed for the precipitation of the total antiphosphorylase content of a serum, with the antigen in excess the same amount of antiphosphorylase will neutralize the activity of $2x$ units of phosphorylase. In other words, as opposed to neutralization with the antigen in excess, phosphorylase is able to unite with a twofold amount of antiphosphorylase at the equivalence point. Thus, the amount of antigen equivalent to the antiphosphorylase content of a serum may be determined by measuring the inhibitory effect in antiphosphorylase units with an excess of antigen. Half of this value will correspond to the equivalent quantity of antigen in phosphorylase units. In this way, quantitative precipitation and determination of the equivalence point are performed easily.

The finding that with the antigen in excess the antiphosphorylase is able to bind a twofold amount of antigen, is analogous to the toxin-antitoxin reaction known as the Danysz phenomenon [7]. When a given amount of diphtheria toxin is added to the amount of antitoxin needed for neutralization not all at once, but in two or more fractions with intervals of 15 minutes or more, the antitoxin is unable completely to neutralize the toxin. The phenomenon was observed also with other toxins [8]. CINADER showed that the Danysz phenomenon was demonstrable in *Cl. perfringens*, where the lecithinase antigen bound twice the amount of antilecithinase sufficient for neutralization. The lecithinase antigen was not crystalline and thus no quantitative precipitation could be carried out. Our experiments performed by a different technique with phosphorylase-antiphosphorylase afford a basis for similar conclusions. The crystalline rabbit muscle phosphorylase allowed quantitative precipitation experiments which showed that in antigen excess due to the formation of soluble antigen-antibody complexes, the amount of precipitate decreased. Nevertheless, the absolute value of inhibition increased indicating that inhibition is not necessarily accompanied by precipitation. The shape of lecithinase-antilecithinase neutralization curve is closely similar to that yielded by the phosphorylase-antiphosphorylase system.

Table I explains the correlation between the enzyme inhibiting action and precipitating antibody content of antiphosphorylase sera. On the average, in sera 1—4 one antiphosphorylase unit was equivalent to 0.016 μ g antibody N. In serum 5, most probably due to the presence of antibodies blocking not exclusively the enzymically active groups of phosphorylase, a more significant difference was obtained. Antibodies acting on other, enzymically inactive, groups of phosphorylase might also have been present. These antibodies were precipitated at the equivalence point, but the complexes formed were dissolved in the excess of antigen and remained enzymically active. The difference in the antibody N content between serum aliquots containing 1 antiphosphorylase unit is probably due to the different proportion of the two kinds of antibody. This proportion is influenced within some limits by individual differences between the animals, but may as well depend on the immunizing doses, manner of immunization, etc. Accordingly, the above-given 0.016 μ g antibody N/1 antiphosphorylase unit can be regarded merely as a value for orientation.

As to the two sera giving neutralization points in the zone of excess antigen, it may be assumed that the avidity of these sera was higher than the average, and the complexes usually characteristic of extreme antigenic excess, were produced even in the case of a moderate excess of antigen.

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PLASMA PHOSPHATASE, CHOLINESTERASE AND PEPTONASE IN ANAPHYLACTIC SHOCK*

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Summary. During anaphylactic shock, plasma phosphatase activity showed in guinea pigs a rise of 111 per cent, in rabbits one of 54.5—63.0 per cent. In rabbits, plasma cholinesterase activity decreased by 30 per cent, while peptonase activity increased by 17 per cent.

H substances liberated as a result of allergic cellular reaction are known to be primarily responsible for the main symptoms of anaphylactic shock. It has been shown that the liberation of histamine is closely associated with the disruption and degranulation of mast cells [1]. Histamine, heparin and serotonin are released from disrupted mast cells by the anaphylactic shock inducing injection or by histamine liberators. In mast cells the presence of certain enzymes, such as alkaline phosphatase, has also been demonstrated [2]. If after the disruption of mast cells heparin is present in the blood, it is justified to assume that other high molecular weight constituents, for instance enzymes, may as well gain access to the circulation. It seemed therefore interesting to investigate the behaviour of plasma phosphatase activity during anaphylactic shock.

Cholinesterase is known to play an important part in the physiological activity of neural and other tissue cells. Therefore, observations concerning the changes in cholinesterase activity might be valuable for understanding the mechanism of neural symptoms met with in anaphylactic shock.

Measurements of plasma peptonase activity were undertaken because in the recent search for the mechanism of anaphylactic shock much attention has been paid to proteolytic products such as the polypeptides [3]. The behaviour of peptonase has also been considered interesting, in view of the observation that heterologous protein injections increase the activity of that enzyme.

Materials and methods

Sensitization of animals and induction of shock. Although the guinea pig is regarded as the most suitable animal for studying anaphylaxis, in view of the large amounts of blood samples needed, the experiments were mainly carried out in rabbits of both sexes weighing 2—3 kg. Part of the animals sensitized to crystalline ovalbumin received on alternate days 2

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intravenous, 2 intramuscular and 1 intraperitoneal injections, each of 2 ml of a 5 per cent antigenic solution. Other animals were injected in a similar manner with native egg albumin. The shock-inducing dose was administered 21–30 days after the last injection.

In order to give rise to a prolonged non-fatal anaphylactic shock, sufficient amounts of 5 per cent crystalline ovalbumin or 40 per cent native egg albumin were injected into the auricular vein of the animals. The 2 ml initial dose was repeated until the first sign of anaphylactic reaction had appeared.

In order to obtain blood samples subsequently, one of the common carotid arteries was prepared in local anaesthesia. Samplings were performed before and 10, 25 and 45 minutes after the administration of the shocking dose. The 3 ml blood samples were taken into tubes containing 0.05 ml 30 per cent potassium oxalate. Prior to alkaline phosphatase, cholinesterase and peptonase determination, the samples were stored at 0° C.

In other experiments guinea pigs weighing 300–400 g of both sexes were used. Sensitization was carried out partly with 1 mg crystalline ovalbumin, subcutaneously. Induction of shock was performed 21 days later. A blood sample was obtained by cardiac puncture and then 4 mg ovalbumin antigen was reinjected into the heart. Immediately after death the guinea pigs were bled by decapitation. The blood was collected in tubes containing potassium oxalate.

Other guinea pigs were sensitized passively with intracardiac injections of anti-ovalbumin rabbit serum containing 70 μ g antibody N. As a shocking dose 4 mg crystalline ovalbumin was given intravenously. After the fatal shock blood samples were collected and examinations were carried out in the blood sera. Each of 10 non-sensitized control guinea pigs received 4 mg ovalbumin intravenously and the sera were used for phosphatase activity assay.

Alkaline phosphatase determination was carried out as described by BODANSKY, with the exception that smaller total volumes were used.

Inorganic phosphorus was assayed by TAUSKY and SCHORR's method [5].

Cholinesterase was determined according to HESTRIN [6]. As a substrate acetylcholine chloride was used at 3 mM concentration.

Peptonase activity was estimated as described by DÁN, NAGY and TÓTH [7], with the modification that by the addition of potassium oxalate, the Witte peptone solution was previously depleted of calcium.

Results

Phosphatase. Phosphatase activity during anaphylactic shock was examined in 20 rabbits and 20 guinea pigs. The average data obtained in rabbits is presented in Fig. 1. It is seen that 10 minutes after the administration of the shocking dose the phosphatase level increased by 1.07 Bodansky units, and remained so throughout the 45 minute experiment. This level corresponded to a 54 per cent rise as compared to the zero minute value (2.0 units). Of the 20 rabbits 9 had been sensitized to crystalline ovalbumin, 11 to native egg albumin. The former group of animals exhibited a higher rise in phosphatase activity.

The plasma phosphatase level of 7 control rabbits did not differ significantly before and after the injection of crystalline ovalbumin. When preincubated *in vitro* with the homologous antigen, the phosphatase activity of anti-ovalbumin rabbit serum was likewise unaltered.

Tables I and II show experiments with guinea pigs. As compared to the 3.19 units mean phosphatase activity measured in non-sensitized animals, the corresponding mean value in passively sensitized animals was 6.83 units (111 per cent rise). Nine out of the 10 sensitized guinea pigs died of acute anaphylactic shock within 5 minutes.

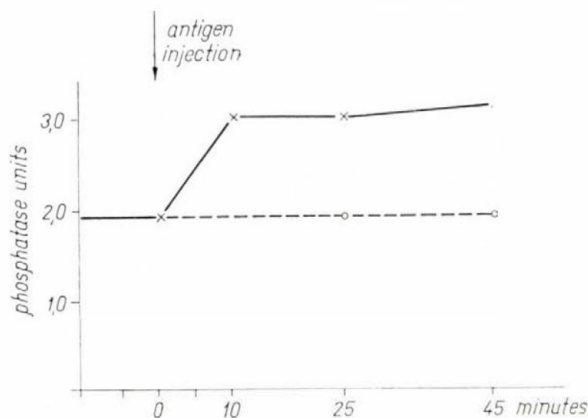


Fig. 1. Plasma phosphatase activity in sensitized and control rabbits. 10, 25 and 45 minutes:
 $p < 0.01$.

○—○ control animals, ×—× sensitized animals

In actively sensitized guinea pigs the plasma phosphatase activity increased by 46.7 per cent after the administration of the shocking dose. Of the 11 animals of this group only 5 died of acute anaphylactic shock within 5 minutes. In the surviving guinea pigs with decreased doses a protracted shock was induced. These animals were bled 30–40 minutes after the administration of the antigen.

Cholinesterase determination was carried out in 18 rabbits. Fig. 2 shows the changes in plasma cholinesterase after the administration of the shocking

Table I

Serum phosphatase activity in control and passively sensitized guinea pigs after the induction of anaphylactic shock

Serum phosphatase in Bodansky units	
Control animals	Sensitized animals
1.5	3.4
1.9	4.2
2.2	6.0
2.8	6.6
2.9	6.9
3.5	7.2
3.7	7.6
4.2	7.8
4.3	9.2
4.9	9.4
Average 3.19	6.83 +111%

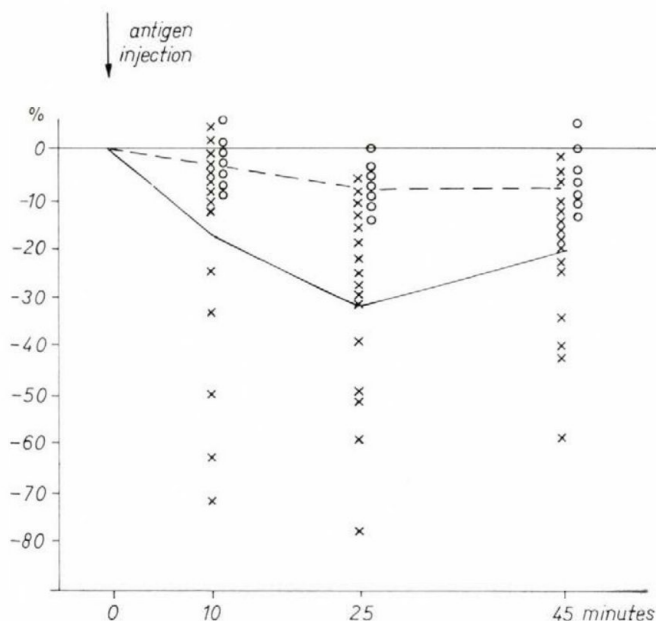


Fig. 2. Plasma cholinesterase activity in sensitized and control rabbits
○—○ control animals, ×—× sensitized animals

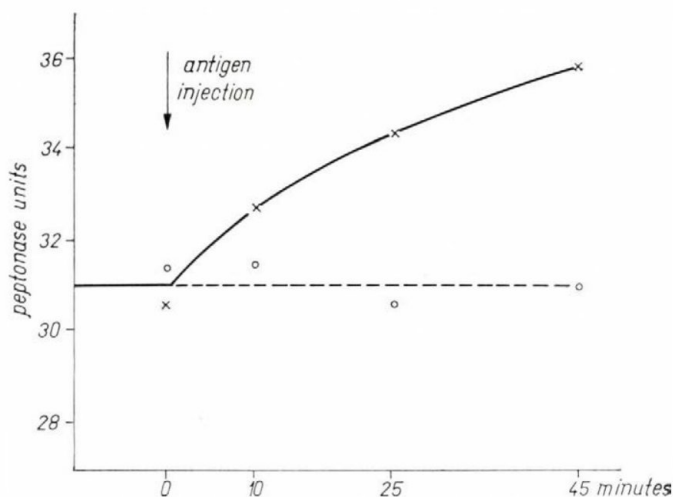


Fig. 3. Plasma peptonase activity in sensitized and control rabbits.
10 min.: $p < 0.10$; 25 min.: $p < 0.02$; 45 min.: $p < 0.01$; ○—○ control animals, ×—× sensitized animals

dose. It can be seen that after 10 minutes cholinesterase activity decreased as an average by 17 per cent. The maximum decrease (—30 per cent) was reached in the 25th minute. In the 45th minute the level increased to —20 per cent of

Table II

Plasma phosphatase activity in actively sensitized guinea pigs before and after the induction of anaphylactic shock

Plasma phosphatase in Bodansky units	
Before injection	After injection
0.6	1.2
3.0	3.6
4.8	4.8
3.6	4.8
4.2	4.8
2.4	3.0
2.2	6.0
1.2	2.4
1.8	2.4
1.2	2.4
0.2	1.2
Average 2.27	3.33 $\pm$ 46.7%

the initial value. In rabbits sensitized with crystalline ovalbumin, the decrease in cholinesterase activity was higher than in animals injected with native egg albumin. In non-sensitized control rabbits the fall in cholinesterase activity following crystalline ovalbumin injection was 8 per cent.

In Fig. 3 the result of peptonase assays is presented. In sensitized animals (17 rabbits) peptonase activity gradually increased, and 45 minutes after the shocking dose it showed a 17 per cent rise. The 5 control (non-sensitized) animals exhibited no increased cholinesterase activity after the injection of the antigen.

Discussion

It has been found that after the induction of anaphylactic shock, plasma phosphatase activity increased in rabbits by 54 per cent, in guinea pigs by 111 per cent. In rabbits the rise was evident 10 minutes after the injection of the shocking dose and the level remained high for at least 45 minutes. Thus, the rise in phosphatase activity after the manifestation of clinical shock symptoms indicates that the change is a consequence of the allergic reaction. More recently it has been described that in allergic reactions biologically active substances (histamine, heparin, serotonin, *etc.*) may gain access to the circulation from disrupted or degranulated mast cells [1]. The rise in plasma phosphatase in anaphylaxis may also be due to the disruption

of mast cells, when their constituents, such as MONTAGNA's phosphatase are released and pass into the blood. In addition to mast cells, other cells may, of course, be the source of phosphatase. In rabbits' anaphylactic shock H substances liberated from blood platelets are of importance. In the osmotic lysis of human thrombocytes, in addition to serotonin, the release of pyrophosphatase, acid and alkaline phosphatase was also noted [8]. Phosphatases may be liberated in thrombocytolysis due to antigen-antibody reaction. Similar observations concerning other serum enzymes have also been made. In the necrosis of heart muscle a considerable rise, proportional to the size of the affected area, occurs in serum glutamic acid-oxalacetic acid transaminase [9]. Aldolase, as well as glutamic acid-oxalacetic acid transaminase activity increases in hepatic lesions [10]. The rise in phosphatase may, on the other hand, be explained by a decreased excretion. Alkaline phosphatase is excreted by the liver with the bile. Thus, in consequence of a decreased excretion, the obstruction of the bile duct may also be responsible for an elevated plasma phosphatase level. In the present experiments, however, the rapid rise and then the constant level of the enzyme suggested an increased release and not a decreased excretion.

In our experiments plasma cholinesterase decreased by 30 per cent in anaphylactic shock. The maximum decrease occurred after the manifestation of the clinical symptoms. In guinea pigs HEIM [11] found that apart from an initial transient rise, the plasma cholinesterase decreased during anaphylactic shock. ENGELHARDT *et al.* [12] claimed that during anaphylactic shock the decrease in the rabbits' plasma cholinesterase level was only 10 per cent; this value was similar to that found in control animals. The discrepancy between these findings and our own observations may be due to that ENGELHARDT *et al.* regarded rabbit plasma cholinesterase as a pseudocholinesterase, despite that according to AUGUSTINSSON the plasma of the rabbit contains real cholinesterase in considerable amounts [13]. As ENGELHARDT *et al.* worked with a substrate concentration 7 times higher than the optimal, in the presence of real cholinesterase a substrate inhibition must have been occurred. It should be noted that erythrocyte cholinesterase, which is a real cholinesterase, showed in the mentioned authors' experiments a 45 per cent decrease during anaphylactic shock.

From experiments *in vivo* and *in vitro* it is probable that the decrease in cholinesterase activity during anaphylactic shock is attributable to the effect of liberated histamine and serotonin [12].

Little evidence has so far been presented to elucidate the origin and significance of plasma peptonase. An increase in serum peptonase activity has been observed during immunization [4, 4a] and in acute and chronic hepatitis [7]. No data apart from our observations, are available for the behaviour of this enzyme in anaphylactic shock. UNGAR *et al.* [3] associated with proteolysis

the liberation of H substances encountered in anaphylactic reactions. After the addition of the corresponding antigen to the serum of sensitized guinea pigs, fibrinolysin is activated for a short period. Subsequently a rapid inactivation of the enzyme takes place.

The present experiments showed a 17 per cent increase in the activity of plasma peptonase after anaphylactic shock. The rise is not significant and there is no reason to associate it with histamine liberation. Although Witte peptone (peptic digest of fibrin) has been used as a substrate, it is improbable that the same enzyme would be responsible for both the small rise in peptonase activity found in the present study and for the fibrinolysin activation described by UNGAR *et al.* Indicating that two different enzymes are concerned, serum peptonase and fibrinolysin levels showed opposite types of behaviour in cirrhosis and acute hepatitis [7].

The rise in peptonase level during anaphylactic shock may be explained as follows. (i) Peptonase is liberated from degranulated mast cells; (ii) Peptonase level increases because of an anaphylactic hepatic lesion; (iii) Anaphylactic shock as a non-specific stimulus causes an increased release of peptonase into the circulation; (iv) Other mechanisms are also possible.

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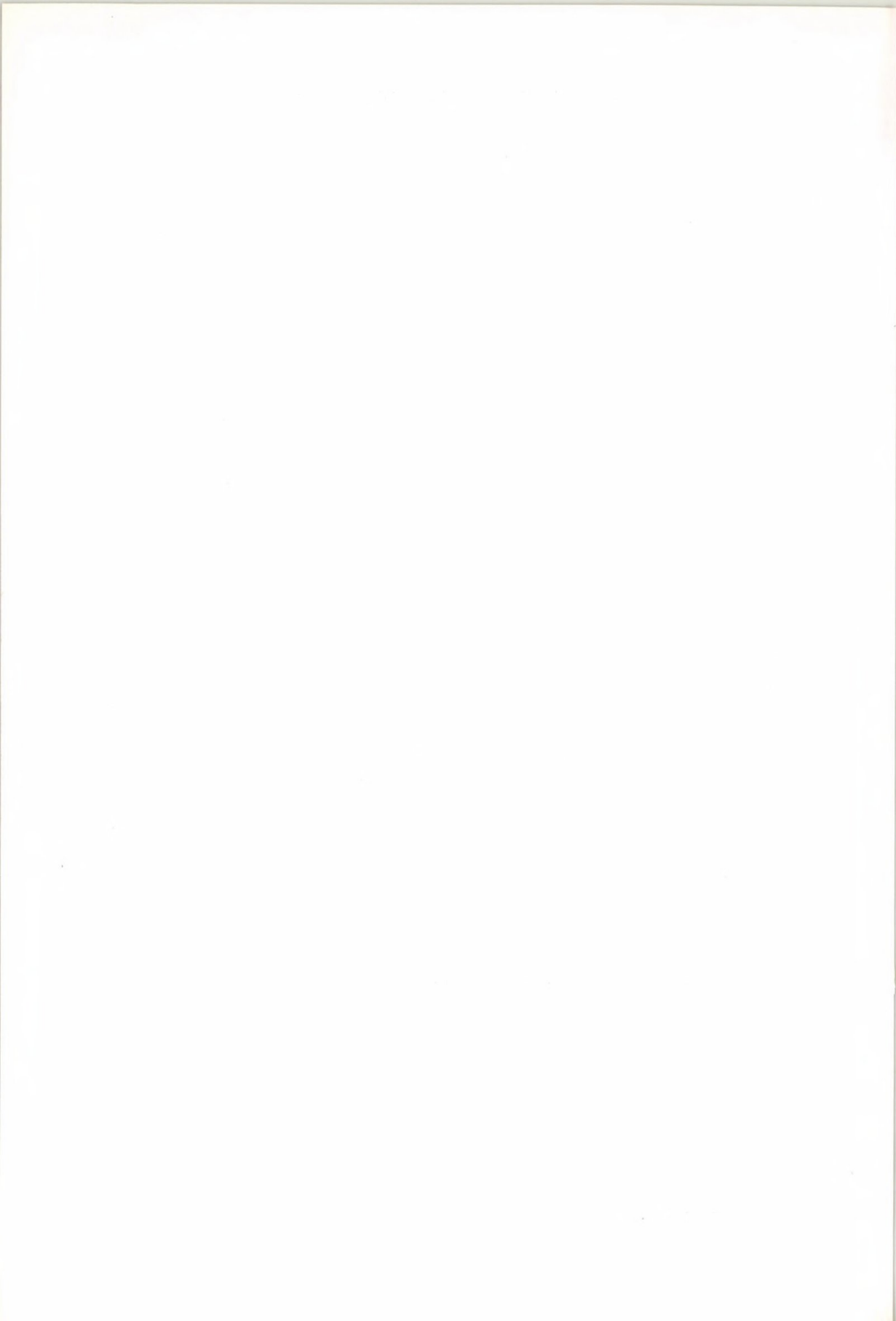
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STUDY OF THE MULTIPLICATION OF VARICELLA- ZOSTER VIRUS BY THE FLUORESCENT ANTIBODY TEST

By

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Summary. The pathological changes and the intracellular location of viral antigen have been studied in human embryonic fibroblast cultures at different times following inoculation with varicella-zoster virus.

The first cytopathic changes were visible 10 hours after inoculation; at this time some minute, eosinophilic granules, each surrounded by a light area appeared. Characteristic type-A inclusions were visible 48–72 hours after inoculation. Complete destruction of cells took 96–144 hours.

Viral antigen was first detectable about the 10th hours of infection; some minute fluorescent spots were seen in the nuclei. In the period from 24 to 48 hours nuclear fluorescence increased and cytoplasmic fluorescence appeared. After the 72nd hour the antigen gradually disappeared from the nucleus while the cytoplasm kept fluorescing.

The intracellular distribution of viral antigen and the formation of type-A nuclear inclusions seem to be parallel phenomena.

The cytopathogenic (CP) effects produced by the varicella and zoster viruses in tissue cultures have been thoroughly studied and summarized by WELLER *et al.* [1]. These phenomena, as we have reported elsewhere [2], are similar to the cellular changes due to herpes simplex virus in many respects; in both cases the first changes appear in the nucleus and lead to formation of type-A nuclear inclusion. The cytoplasmic changes are less prominent.

The objects of the present investigations have been to observe the sequential development of the CP changes, and to follow the formation of viral antigen by the fluorescent antibody technique, in tissue cultures infected with the varicella-zoster virus.

Materials and methods

Tissue cultures. Trypsinized human embryonic fibroblasts were cultivated on cover-slips in a medium consisting of 15 per cent bovine serum, 15 per cent bovine amniotic fluid, and 70 per cent Hanks' balanced solution in which 0.5 per cent lactalbumin hydrolysate and 0.075 per cent NaHCO_3 had been dissolved (pH 7.2). The medium contained phenol red, as indicator, and 600 units of penicillin and 400 μg of streptomycin per ml. The cultures were prepared in small Roux flasks, in each of which two cover slips of 18×18 mm had been placed. The cover-slip cultures were inoculated when the monolayer had developed.

Inoculation of tissue cultures. The varicella virus strain under study had been isolated by us [2] and had undergone 30 passages in human embryonic fibroblast cultures. As inoculum, fibroblasts obtained from cultures showing specific, diffuse CP pictures were used. These were dispersed by digestion in 0.25 per cent trypsin, centrifuged, and suspended in the maintenance fluid. Aliquots of this suspension containing approximately 15,000 cells each were added to the cover-slip cultures. These were then incubated at 37°C in the maintenance medium being differ-

ent from the growth medium in containing 20 per cent bovine serum and no bovine amniotic fluid.

Examination of infected tissue cultures. Cover slips were removed at intervals (2, 10, 24, 48, 72, 96 and 144 hours after inoculation) and washed twice in an isotonic buffer solution (PBS) containing sodium chloride and 0.02 M phosphate buffer of pH 7.4. Some of the cultures were fixed in BOUIN's solution and stained with haematoxylin and eosin (H-E), the others were fixed in acetone for 10 minutes at room temperature and examined by the fluorescent antibody technique.

Fluorescent antibody technique. The indirect method was employed. The preparations were covered with 1 in 10 dilution of a human convalescent serum and allowed to stand at room temperature in the wet box for 30 minutes. The convalescent sera were taken from herpes-zoster patients at least two weeks after the onset of illness. (According to WELLER's [3] and own observations [4], herpes zoster and varicella convalescent sera give cross reactions with each other). After being washed in PBS for an hour the cover slips were overlaid with a solution of fluorescein-isothiocyanate-conjugated* [5] equine anti-human gamma globulin and incubated at room temperature for 30 minutes. After another washing with PBS for an hour the preparations were covered with buffered (pH 7.0) glycerin and examined under the fluorescence microscope.

Control preparations (i). Noninfected human fibroblastic cultures were treated in the same way; (ii) normal bovine serum was substituted for the human convalescent serum; (iii) as conjugate equine diphtheria-antitoxic gamma globulin was added to preparations instead of anti-human gamma globulin. The specificity of conjugates was checked by absorption with powdered mouse liver (2×100 mg per ml) [6] and with trypsinized human fibroblasts (2×4 million per ml).

Fluorescence microscopy. Zeiss HBO 50 mercury-vapour lamp was used as source of UV light. As exciter filter UG 1/3.5, as ocular filter GG9 were applied. Photos were prepared in Agfacolor negative UT films (17/10 DIN). The time of exposure varied from 10 to 60 seconds.

Results

The pathohistological changes following inoculation with varicella virus can be summarized as follows.

Up to the 10th hour no changes are visible except in the cells of inoculum origin that had attached to the monolayer. These cells show various degrees of CP changes (Fig. 1).

From the 10th hour on CP changes appear in cells neighbouring the inoculum cells. Various degrees of nuclear rarefaction and eosinophilic granulation in the centres of rarefied areas appear (Fig. 2).

In a later stage foci involving more and more damaged cells are visible. The cells in the central area of each focus show a more progressed stage of the CP process than the peripheral cells, in accordance with the centrifugal, from-cell-to-cell spread of infection. As a result of confluence of eosinophilic granules smaller or larger spots appear in the nuclei of the central cells; the chromatin forms gross granules and is gradually shifted to the margin of the nucleus. Subsequently a characteristic, type-A nuclear inclusion surrounded by a light area develops and the chromatin is aggregated immediately under the nuclear membrane. In this stage most of the nucleoli disappear (Fig. 3). Polynuclear giant cells are also common (Fig. 4).

* The isothiocyanate kindly was supplied by the Baltimore Biological Laboratory, Baltimore 18, Maryland, U. S. A.

Figs. 1—5. Human embryonic fibroblast cultures at different times after inoculation with varicella-zoster virus. Haematoxylin and eosin stain

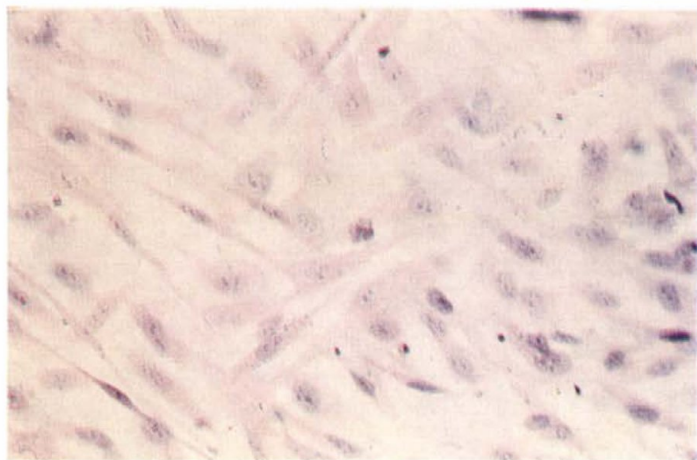


Fig. 1. 2 hours. A greatly shrunken cell of inoculum origin with type-A intranuclear inclusion at the centre of an intact culture
Magnification, $\times 500$

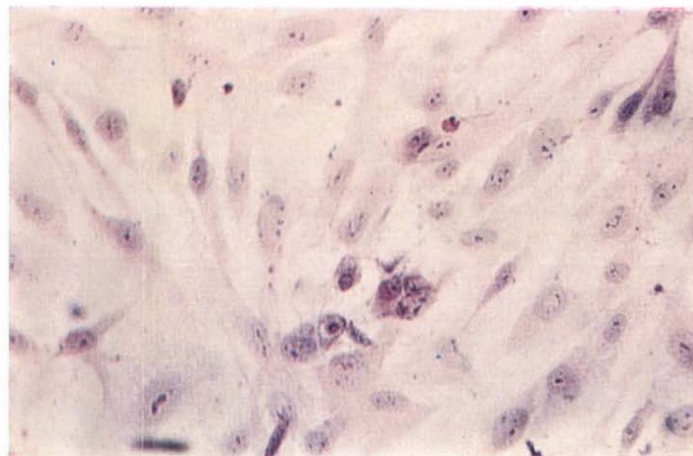


Fig. 2. 10 hours. Initial nuclear change in cells neighbouring inoculum cells. Magnification, $\times 500$

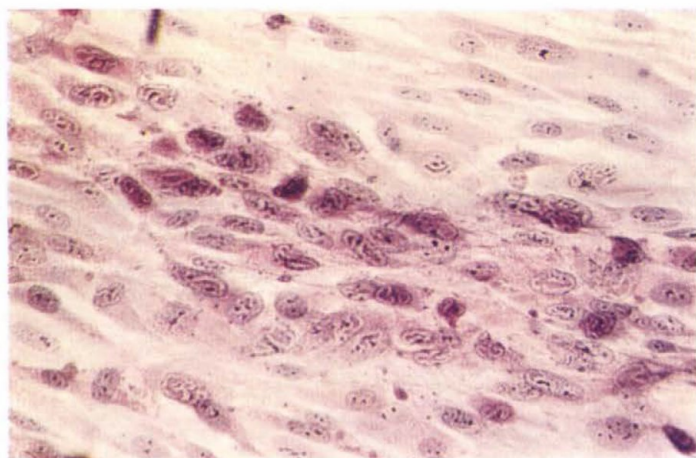


Fig. 3. 72 hours. Cytopathic focus. Type-A nuclear inclusions; cells at the periphery of the focus show earlier stages of the pathological change. Magnification, $\times 500$

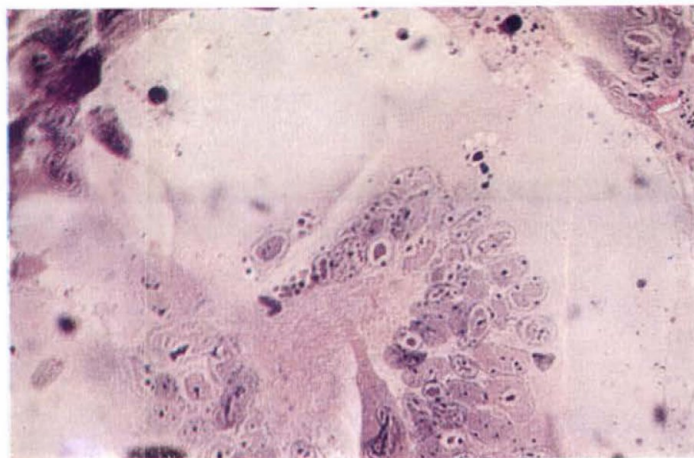
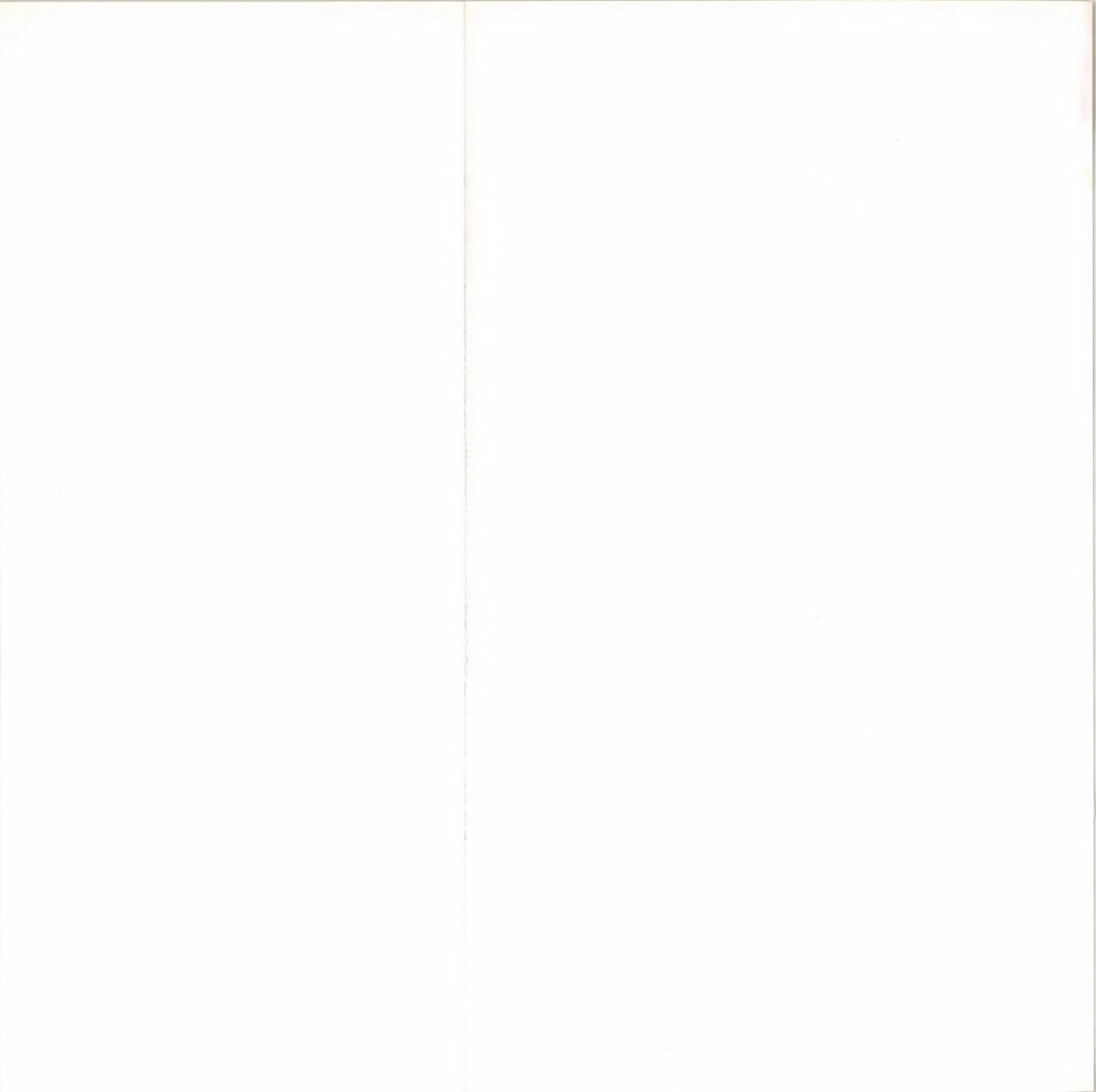


Fig. 4. 72 hours. Giant cell. Magnification, $\times 500$



Figs. 6—10. Human embryonic fibroblast cultures at different times after inoculation with aricella-zoster virus. Fluorescent antibody technique

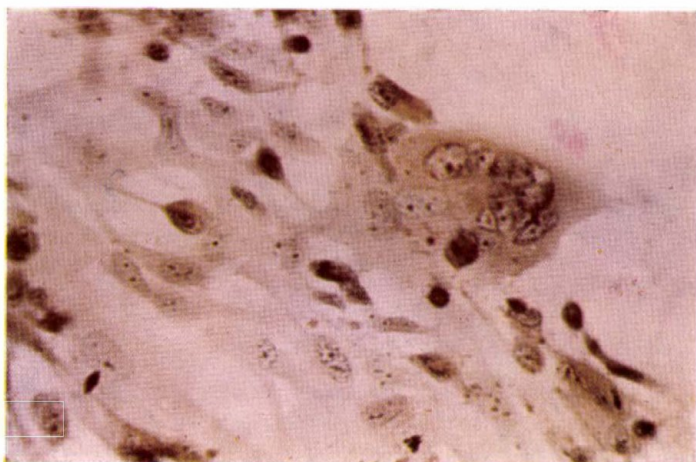


Fig. 5. 96 hours. Some type A inclusions are still visible, most of them have disappeared. Magnification, $\times 500$

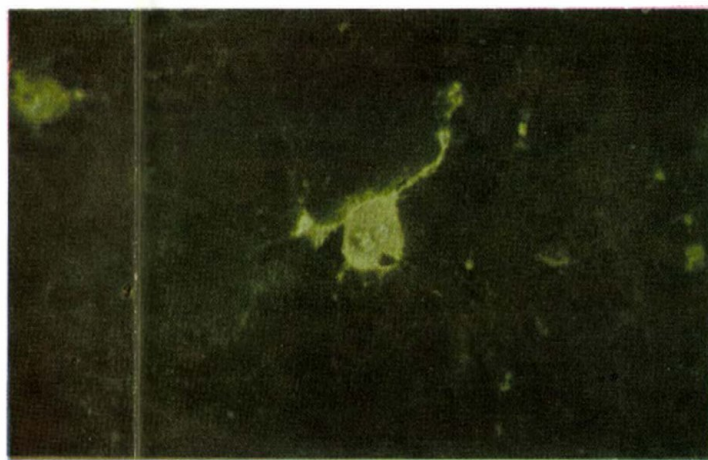


Fig. 6. 2 hours. A single cell of inoculum origin. Magnification $\times 500$

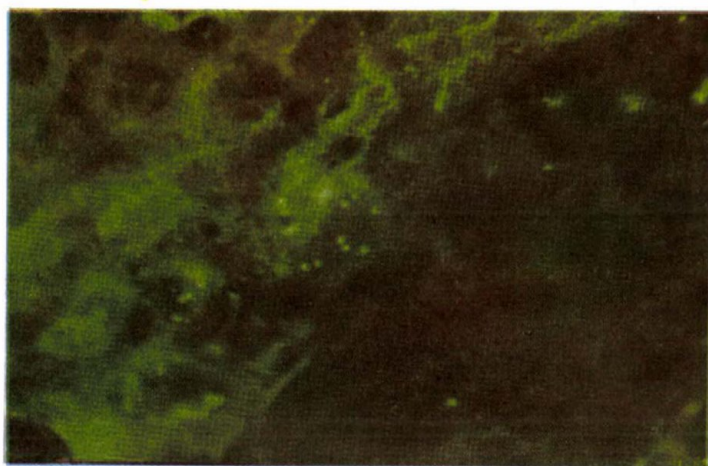


Fig. 7. 10 hours. First appearance of intranuclear viral antigen at the periphery of a focus. Magnification, $\times 1000$

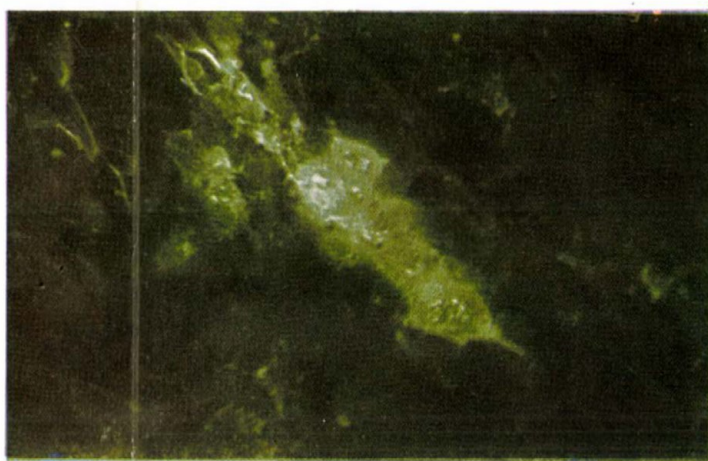


Fig. 8. 24 hours. A large part of the nucleus is already filled by the viral antigen. Fluorescence appears in the cytoplasm as well. Magnification, $\times 500$

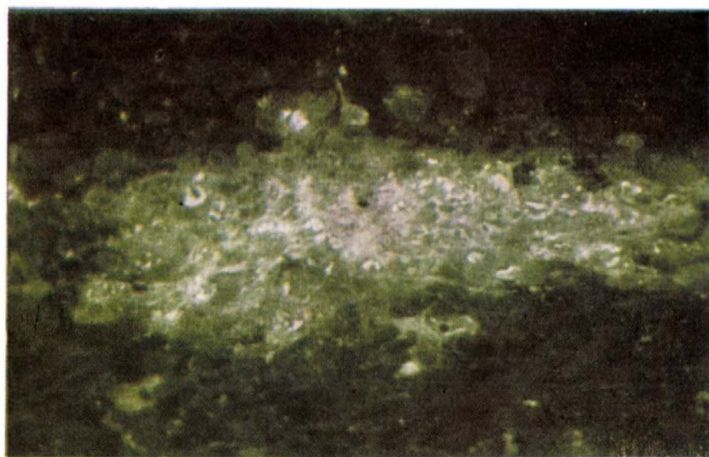


Fig. 9. 48 hours. Both the nuclei and cytoplasms are filled by viral antigen. Magnification, $\times 500$

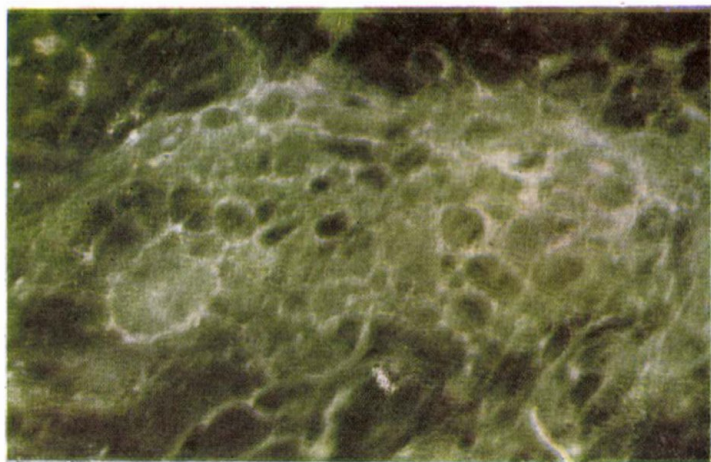


Fig. 10. 72 hours. Viral antigen has, partly or completely, disappeared from the nuclei. Magnification, $\times 500$



At the final stage cell degeneration is more pronounced, remnants of chromatin are recognizable in the form of irregular, basophilic granulation, in which occasional residues of the inclusion are seen; the cytoplasm is replaced by eosinophil-granulated amorphous debris (Fig. 5).

Changes shown by the fluorescent antibody method. Up to the 10th hour after inoculation the inoculum cells are well-recognizable by their bright fluorescence (Fig. 6).

As the first signs of infection, some minute, intranuclear granules appear in some neighbouring cells (Fig. 7). Subsequently the cytoplasm is gradually filled by viral antigen (Fig. 8) and the intranuclear granules grow until almost the whole nucleus is filled by a brightly fluorescent mass. At this stage, when numerous cells are already affected, a bright, diffuse fluorescence is visible in the cytoplasm as well (Fig. 9). At a later stage nuclear fluorescence weakens and, finally, disappears; specific fluorescence is shown only by the cytoplasm (Fig. 10).

In spite of the pathomorphological difference between central and peripheral cells, both methods show the predominance of a well-defined stage of CP process in the majority of the cells within each focus. In Table I we have attempted to illustrate the temporal sequence of CP changes.

Temporal sequence of CP changes. In Table I the CP changes observed on staining with HE are classified into three arbitrary stages.

Stage I is characterized by initial signs of CP changes, without type-A nuclear inclusions; Stage-II cells have a well-developed type-A inclusion each, while more or less destroyed cells are classified into Stage III. In the latter cells hardly any or no inclusions are detectable.

CP foci were characterized by the stage of the majority of the cells forming them.

At the time of taking sample the foci visible in 2—4 parallel cover-slip cultures were counted and their distribution by stage was established.

Scattered initial changes occurred as soon as at the 10th hour, but the majority of these appeared later, about the 24th hour. Well-developed nuclear inclusions became first visible at 48 hours and the maximum incidence of stage-II foci was attained at 72 hours. Complete destruction of cells started at the same time and became more intensive subsequently.

Temporal sequence of the appearance of viral antigen. This process in the function of time is presented in Table II in a similar manner as the CP process was shown in Table I.

In Table II Stage I means the stage when viral antigen can only be found in the nucleus; at Stage II both the nucleus and the cytoplasm contain antigen; at Stage III the antigen disappears partly or completely from the nuclei.

CP foci were characterized, here too, by the stage to which the majority of the cells belonged.

Table I

Stages of cytopathic changes caused by the varicella-zoster virus

Stage*	No. and percentage distribution of CP foci at various times							
	2 hours No. per cent	10 hours No. per cent	24 hours No. per cent	48 hours No. per cent	72 hours No. per cent	96 hours No. per cent	144 hours No. per cent	
Stage I	0 0	12 100	48 98	93 75	16 14	0 0	0 0	
Stage II	0 0	0 0	1 2	30 25	78 68	124 56	26 42	
Stage III	0 0	0 0	0 0	0 0	21 18	99 44	36 58	
Total foci**	0 0	12 100	49 100	123 100	115 100	223 100	62 100	

* Stage I: initial nuclear changes

Stage II: well-developed type-A inclusions

Stage III: destruction of cells

Detailed explanation in the text

** Sum of CP foci in 2—4 parallel cover-slip cultures.

First traces of antigen were visible 10 hours after inoculation; Stage-II cells had reached a considerable number by 24 hours. Nuclei became most saturated by antigen between 24 and 48 hours; in the same period antigen

Table II

Stages of appearance of intracellular viral antigen in varicella-zoster-virus-infected cells

Stage*	Number and percentage distribution of CP foci at various times after infection							
	2 hours No. per cent	10 hours No. per cent	24 hours No. per cent	48 hours No. per cent	72 hours No. per cent	96 hours No. per cent	144 hours No. per cent	
Stage I	0 0	18 100	23 23	1 1	0 0	0 0	0 0	
Stage II	0 0	0 0	77 77	70 90	22 49	21 26	3 5	
Stage III	0 0	0 0	1 1	7 9	23 51	61 74	55 95	
Total foci**	0 0	18 100	101 100	78 100	45 100	82 100	58 100	

* Stage I: viral antigen only in the nucleus

Stage II: both the nucleus and the cytoplasm contain antigen

Stage III: viral antigen cannot or can hardly be detected in the nucleus

Detailed explanation in the text

** The sum of CP foci in 2—4 parallel cover-slip cultures.

appeared in the cytoplasm. Nuclear fluorescence began to diminish in the period from 48 to 72 hours. At 96 and 144 hours there was hardly any fluorescence in the nucleus visible; only the cytoplasm contained antigen.

Discussion

WELLER and COONS [3] studied the varicella virus by the fluorescent antibody method as early as 1954, but the object of their investigations was, first of all, to demonstrate immunological cross reactions between convalescent sera of patients with varicella and those with herpes zoster; they did not register the accurate intracellular location of viral antigen. For the herpes simplex virus, LEBRUN has demonstrated [7] that at the beginning the antigen is detectable only in the nucleus, later in both the nucleus and the cytoplasm, and finally only in the cytoplasm. According to LEBRUN's data, the whole process takes about 72 hours.

The present studies have shown that the first appearance of the viral antigen in cells infected with varicella-zoster virus is demonstrable in the nucleus, in the form of tiny spots. These spots show a gradual growth and confluence until they fill almost the whole nucleus. During the same period the cytoplasm also becomes filled by viral antigen, so that in a certain stage both the nucleus and the cytoplasm seem to be saturated. Subsequently, the quantity of the intranuclear antigen begins to diminish and, at last, viral antigen is only detectable in the cytoplasm. These observations conform with those of TOURNIER *et al.*, [8], who followed the multiplication of this virus in human fibroblasts by electron microscopy.

The CP changes shown by the varicella-zoster virus are in certain respects different from those produced by the herpes simplex virus; *e.g.* the CP changes caused by the former remain of focal character through the whole process while the herpes-simplex lesions tend to become diffuse. Nevertheless, based on the appearance and intracellular distribution of the varicella-zoster and herpes simplex antigens (for the latter see LEBRUN's [7] data), the course of the reproduction of both viruses seems to be very similar to each other. This statement may serve as a new basis for the taxonomy of these viruses, taking into account that the electron microscope shows the two viruses to be similar [9].

The question whether the antigen demonstrable in the nucleus by the fluorescent antibody technique is the complete antigen of the virus or represents only an antigenic component like in the case of certain related [10] and unrelated [11] viruses, requires further research.

The H—E-stained preparations show that the first changes, *viz.* fine eosinophilic granules, appear in the nucleus soon, 10 to 24 hours, after inoculation with varicella-herpes virus. Subsequently these granules gradually increase to form a characteristic type-A inclusion. We have made

attempts to establish whether these inclusions do or do not contain viral antigen. LEBRUN suggests that the disappearance of virus antigen from the nucleus precedes the formation of nuclear inclusion in herpes-simplex-infected cells. In the present case the elucidation of the question is hindered by two circumstances.

(i) We could not demonstrate nuclear inclusions in preparations that had been fixed in acetone and examined by the immunofluorescence technique. On the other hand, the stained preparations were not suitable for the fluorescence antibody method.

(ii) We could never obtain identical preparations for comparative examination with the two methods, for every focus showed cells of different stages regardless whether it was examined for CP changes or viral antigen. The fact that one stage was always predominant, allows only a crude statistical evaluation to demonstrate simultaneity.

It is nevertheless noticeable (Tables I and II) that most of the type-A inclusions appeared at about the time (72 hours) when the bulk of the intranuclear antigen was disappearing. It can therefore be assumed that in the cells infected by the varicella-zoster virus, like in herpes-simplex-virus-infected cells, the nuclear inclusions are formed almost simultaneously with the disappearance of nuclear antigen. However, a considerable number of type-A inclusions were already visible at 48 hours, *i.e.* at a time when nuclear antigen had hardly begun to disappear. We assume, therefore, that at the initial stage of development the nuclear inclusion may contain viral antigen.

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CONTROL OF INFECTIOUS DISEASES WITH REGARD TO ECONOMICALLY UNDERDEVELOPED COUNTRIES*

By

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(Received February 28, 1963)

Survey and analysis of existing conditions

1. A decrease in the incidence of infectious diseases is a world-wide phenomenon. In culturally advanced countries, 30 years ago infectious diseases ranked second and third among the causes of death, and the mortality rate due to infections was 1.5 to 2.5 per 1000 population. Infectious diseases occupy now the seventh or eighth place and are responsible for no more deaths than 0.3 to 0.4 per 1000 population.

2. Although for the past 30 years both the incidence of infectious diseases and mortality due to them have considerably decreased in the economically underdeveloped countries, the decrease is different from that in advanced countries both quantitatively and qualitatively.

(a) *Quantitative changes.* The decrease as observed in the underdeveloped countries means a drop from extreme backwardness to a somewhat better level which still indicates deplorable conditions. The death rate due to infectious diseases dropped in Egypt from 1.34 per 1000 in 1935 to 0.73 per 1000 in 1958; an undoubtedly notable decrease. Still, the rate is the double of the corresponding figure in France, Czechoslovakia or Hungary (Table I).

WINSLOW, relying on data from the time immediately preceding World War II writes the following [1]. While per capita income amounted to \$ 554.— in the United States and \$ 468.— in the United Kingdom with the corresponding life expectancy of 64 and 62 years, respectively, in countries with low per-capita incomes these figures were, Mexico \$ 61.—, 37 years; Brazil \$ 46.—, 39 years; India \$ 34.—, 27 years.

The high rate of infant mortality is indicative of the same trend.

Training of the medical and nursing staff is equally far from satisfactory. In Table II we quote the data of *Santé du Monde* [2] regarding the ratio of medical staff and population.

* Presented at the United Nations Conference on the Application of Science and Technology for the Benefit of the Less Developed Areas, Geneva, February 13, 1963.

Table I

Overall mortality, and death rate in respect of infectious diseases in some countries, for 1935 and 1958

Country	Year	Total number of deaths	Number of deaths due to infections	Rate of deaths due to infections
		per 1000 inhabitants		per cent
Hungary	1935	15.34	2.34	15.3
	1958	9.93	0.43	4.3
Czechoslovakia	1935	13.49	1.99	14.8
	1958	9.34	0.44	4.7
France	1935	15.70	1.65	10.5
	1958	11.15	0.33	3.0
Egypt	1935	25.10	1.34	5.3
	1958	19.68	0.73	3.7
Ceylon	1958	9.67	0.66	6.8
Thailand	1958	10.35	1.51	14.6

The ratio in respect of hospital beds is still worse. For example, in Africa, in the former colonies, the number of beds is 1.7 per 1000 inhabitants [3]. It follows that these countries have hardly any public health administration, medical service, epidemiological organization, *etc.* They will have to develop them in the immediate future, so that it is of primary importance that the manner in, and the methods with, which they will carry out these tasks, be up to the mark.

(b) *Qualitative factors.* Epidemiological conditions in underdeveloped countries are unsatisfactory not only quantitatively, *i.e.* as regards morbidity and mortality, but also in their qualitative aspects. Infectious diseases that have long ceased to constitute a serious problem in economically advanced

Table II

Countries with satisfactory medical supply			Countries with unsatisfactory medical supply		
1 physician					
Soviet Union	per 500 inhabitants		India	per 5,000 inhabitants	
Czechoslovakia	..	590 ..	Afghanistan	..	58,000 ..
Austria	..	620 ..	Mali	..	80,000 ..
Hungary	..	636 ..	Nigeria	..	96,000 ..
U. S. A.	..	790 ..			
France	..	930 ..			

countries are still epidemic in underdeveloped areas. According to statistical data collected by the W. H. O. for 1957—1960, which refer to 33 per cent of the world's total population, infectious enteral diseases, pneumonia and influenza are still the most common causes of death in economically underdeveloped countries. Enteral infections represent a serious problem and they are responsible for the death of 1 per cent of the children under 5 years in those areas.

Let us note in passing that the countries in question are — though to a decreasing extent — still foci of smallpox, cholera, plague, leprosy, trachoma and a number of characteristically tropical diseases. We do not propose to dwell on them in this paper; defensive measures have already been taken by the W. H. O. which, with the cooperation of local institutions, has instituted adequate campaigns to control such diseases.

Methods of control of infectious diseases

Various methods of fight against epidemics have been developed and became firmly established in the hygienically advanced countries since the last third of the 19th century, since the great microbiological discoveries. They have led to the present satisfactory situation. Epidemiological methodology especially since World War II has developed along two sharply different lines. Followers of the one trend — represented mainly by the capitalistic countries of the West — regard prevention vaccination as the primary task of epidemiological work. Nevertheless, even vaccination is not everywhere compulsory because a "compulsion" of this kind might, according to notions prevailing in capitalistic countries, violate civic rights. Adherents of this school look upon the fight with non-specific means (*e.g.* improvement of communal or social hygienic background) as a secondary and subordinate measure. Followers of the other trend, *i.e.* of the method adopted by most socialistic countries, while relying on a system of compulsory, widely applied, gratuitous prophylactic vaccinations, advocate at the same time measures of non-specific protection and consider the latter to be equivalent to vaccination. It seems worth while to compare the two methods, since the superiority or inferiority of the one or the other may decide the nature of assistance to be granted to economically underdeveloped countries.

We propose to measure the efficacy of the given method by its effect on typhoid fever morbidity and mortality during the last 30 years. Typhoid fever is a disease that can be controlled well by specific means, *i.e.* vaccinations, and by non-specific epidemiological measures alike (it is especially sensitive to communal hygienic influences).

Let us now examine the conditions regarding typhoid fever in Hungary during three decades. A study of the development in Hungary is instructive because here both methods have been employed successively.

Vaccination was predominant in Hungary between the two world wars, to be succeeded after World War II by the adoption of the complex method, *i.e.* simultaneous application of specific and nonspecific measures (Fig. 1).

Typhoid fever still meant a serious epidemiological problem in Hungary between the two world wars; the average annual morbidity was 12,000 with about 600 fatal cases. In contrast, in 1960 the number of typhoid cases was

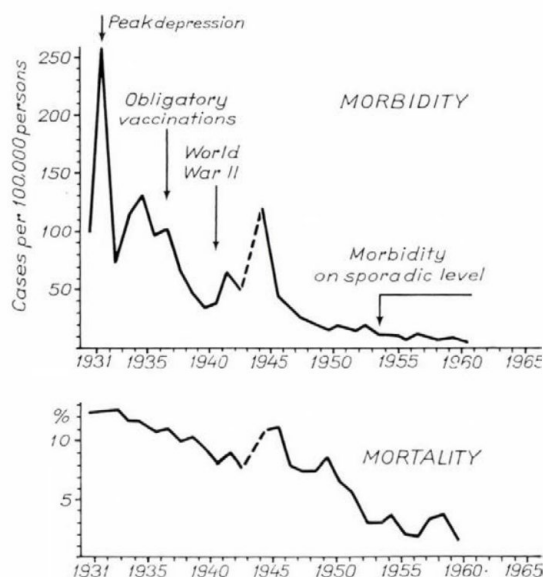


Fig. 1

not more than 500, with only 10 fatalities. What were the factors responsible for this radical qualitative and quantitative change? They are methodological factors. A careful scrutiny of the curve will bear this out. Conditions in respect of communal and social hygiene deteriorated in 1932 (the year when the economic world crisis had reached its peak) with a corresponding jump of the rate of morbidity from the average annual figure of 12,000 to 22,000. Vaccination was introduced in 1937. It can be seen from the curve that the result of this measure was considerable but fairly unstable. Conditions of communal and social hygiene deteriorated once more after the outbreak of World War II, and this nonspecific factor again sent the course of the curve upward.

A permanent control of typhoid fever was achieved after the war only when prevention by vaccination had been supplemented by nonspecific measures, *i.e.* the improvement and control of communal and social hygienic conditions. Apart from quantitative improvement, the situation in respect of typhoid fever, always fluctuating before, became stable thenceforward. The value and

efficacy of the combined method is clearly shown by the increased rate of progress since 1945; the tendency towards further improvement is more marked than either in Great Britain or Finland. While the rate of progress remained stationary in these countries 6.1 per cent and 10.7 per cent per annum, respectively, where prophylactic methods have not changed since the termination of World War II, the introduction of the new combined method has enhanced

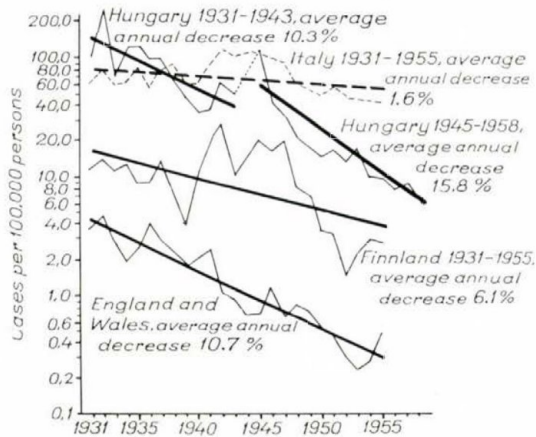


Fig. 2

the rate of improvement in Hungary both quantitatively and qualitatively. The rate of progress was 10.3 per cent before and has risen to 15.8 per cent since World War II (Fig. 2).

Classification

In modern usage infectious diseases are classified according to the point of attack of the pathogen, a method of classification which — on the strength of practical considerations — deserves to be further developed. While observations made during recent years leave no doubt that a combination of specific and nonspecific prophylactic measures has multiplied the efficacy of epidemiological efforts, there are still certain differences in respect of details, and up-to-date classification pays due regard to them. Let us adduce a few examples. It is generally recognized that certain enteral diseases respond well to nonspecific measures. They are, however, more sensitive to hygienic factors of the environment and habitation than to those of social hygiene. We refer in this respect to what we have said above in connection with typhoid fever. Respiratory infections, with their easy spread, are best controlled by vaccination, while nonspecific measures play a secondary role in this respect. On the other

hand, in chronic processes, such as tuberculosis, nonspecific protection (factors of social hygiene in particular) may be of decisive importance. This has to be emphasized because there is a number of authors who — encouraged by the apparently diminishing gravity of the problem of tuberculosis — are of the opinion that tuberculosis can be definitely conquered by chemotherapeutic means alone, *i.e.* without having to resort to expensive nonspecific communal and social hygienic measures (Fig. 3).

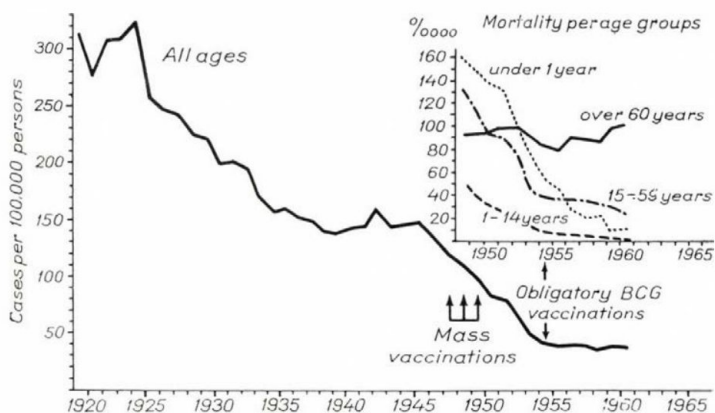


Fig. 3

A successful control of tuberculosis — especially in areas where it still constitutes a grave local problem — is impossible without an adequately improved living standard, the adoption of social insurance on a large scale and the establishment of specific prevention (BCG). Such was the situation in prewar Hungary, and this is the situation now in Brazil (rate of mortality 76.8 per 100,000 population) or Chile (rate of mortality 60.7 per 100,000 population) as also in the economically underdeveloped countries of Asia and Africa.

A combination of specific and nonspecific epidemiological methods has brought excellent results in the fight against haematogenic infections. Specific protection (this being in the cases of malaria a causal specific preventive therapy because it interrupts the chain of infection) applied simultaneously with nonspecific measures (sanitation, elimination of mosquito foci) have yielded permanent quantitative results.

A practical classification, based on the above considerations, distinguishes three groups of diseases.

Group I. This comprises affections in respect of which a satisfactory permanent result can easily be reached by applying a combination of specific and nonspecific measures, *i.e.* the method of preventive vaccination and the

improvement of communal and social hygienic conditions. Whether specific or nonspecific measures should be dominant in a given case will depend on the actual disease to be controlled. Diseases of this category, by being fought in this manner, can be reduced to the level of sporadic occurrences. Typhoid fever is the most typical representative of the group.

To *group II* belong affections against which specific measures — vaccination — have to be employed in the first place, and nonspecific measures in the second place. Respiratory infections are the principal examples of this category, such as diphtheria, whooping cough, *etc.* The reduction of the number of persons per dwelling room, improvement of personal sanitary conditions and of hygienic culture are the nonspecific factors in such cases. It has already been noted that nonspecific factors may become decisively important in the case of chronic processes, notably in that of tuberculosis.

Group III contains affections against which specific prevention has not yet been elaborated, so that nonspecific epidemiological measures are the only means to check their diffusion. This group includes enteral diseases such as dysentery and hepatitis, certain infections of the respiratory tract, such as pandemic influenza, certain recently discovered viral diseases, as also diseases due to hospitalism, such as staphylococcosis, *etc.*

It should be noted in this connection that hepatitis and dysentery have now become increasingly grave urban problems also in hygienically advanced countries, owing to the rapid growth of towns (industrialization). The situation in respect of these diseases, instead of improving shows a tendency to deterioration, because increasing industrialization means increasing demands of water, and difficulties of water supply may give rise to the said diseases and their propagation in densely populated cities and in large industrial plants.

Conclusions

The facilities available for economically and hygienically underdeveloped countries are then the following.

1. Having become independent, they are free to select the best methods when laying the foundations of their general economic policy and determining the general principles of their public-health policy. They can introduce methods that have yielded the best results in hygienically advanced countries, *viz.* the method of prophylactic vaccinations combined with aspecific protection by means of improved conditions of communal and social hygiene.

2. The above-discussed new practical classification of infectious diseases makes it possible to adjust the method to the condition to be controlled, a facility which cannot fail to improve epidemiological results in respect of diseases (*e.g.* typhoid fever), which still constitute a grave problem in certain areas and to reduce such diseases to the level of sporadic occurrences.

Agenda

1. A precise analysis of hygienic conditions, including the detailed examination of the actually existing infectious diseases, and extending to an analysis of all components.

2. The establishment of efficient prophylactic and therapeutic epidemiological medical service, and the training of the necessary expert staff, in all grades. Availability of vaccines by way of imports or production. Establishment of isolation hospitals and laboratories. Gradual construction and development of communal hygienic sanitary institutions, water supply, canalization, refuse disposal, improvement of personal hygiene, serving as bases of non-specific prophylaxis. It is imperative to take all these measures and make all these preparations at a time of beginning industrialization in the economically underdeveloped areas. Adequate water supply, the disposal of industrial sewage are of vital importance for the inhabitants of growing towns, and it must be accompanied by the establishment of social insurance, dispensing of drugs free of charge, *etc.*

3. The realization of the said tasks in accordance with the economic capacity, and their incorporation in the economic programme, of the concerned countries.

4. Preparation of schedules concerning the assistance to be given by economically advanced countries. Assembly of complex work groups composed of experts trained in the field of epidemiology, communal hygiene, social hygiene and sanitary engineering, specifying the burdens that can be carried by the countries concerned, and definition of the tasks that cannot be solved without international assistance. Let us add in this connection that the financial aspects of the two different (specific and nonspecific) methods discussed in the foregoing are different. Vaccination alone is considerably less expensive, but its results are less satisfactory and less permanent than those of the combined method. The simultaneous application of specific and nonspecific measures takes more time and means a heavier financial burden. This, however, must not prevent the adoption of the combined method.

(a) Viewed from the angle of national economy, expenses for the permanent control of epidemics on a large scale are investments which can be amortized in a short time.

(b) Compared to general expenditures necessary at the present technical level of the world, the costs required for up-to-date sanitary measures are not especially high. Let us remember that, according to the statistics of the W. H. O. \$ 1,700 million would suffice for a complete extermination of malaria from the Earth, while about \$ 120,000 million were spent by the nations of the world for armaments in 1962 alone.

A rapid improvement of epidemiological conditions in the economically underdeveloped countries is, thus, no utopia; it can be realized if the economi-

cally more advanced countries succeed in establishing international spiritual and financial cooperation, a competition not aimed at obtaining local advantages but at obtaining the best possible results for the hygienic welfare of countries where local possibilities and means are still weak and national economy is still in the incipient phase.

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HEAT RESISTANCE OF AEROCOCCUS VIRIDANS (WILLIAMS)

By

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(Received February 20, 1963)

Summary. The characteristic indices of heat tolerance (D and z values) have been found considerably higher for *Aerococcus viridans* than for the vegetative forms of other bacteria. From data of heat resistance and penetration, the heat-treatment necessary for preserved products has been determined. It was found that, taking into consideration the destruction of *A. viridans*, commercial products are subjected to a 2 to 3-fold overprocessing.

The importance of *Aerococcus viridans* in commercial food microbiology has become increasingly evident. In a previous paper [1] it has been pointed out that the microorganism is easily mistaken for the hygienically important *Streptococcus faecalis*. In the meat industry, *A. viridans* is a common contaminating bacterium and its greening effect on meat pigment is well-known [2, 3].

As *A. viridans* causes the greenish discoloration of pickled and cooked meats, such as ham products, its heat tolerance is a problem of practical importance. Differential-diagnostically the question is also relevant, as the characteristics of the organism's heat tolerance may help in its identification. As the presence of *A. viridans* is an indicator of the quality of preserved meat products, it seemed desirable to investigate its resistance to the processing temperatures usually applied.

Materials and methods

Heat resistance testing and processing experiments were carried out as described by VAS [4]. The equation for the thermal death time curve was determined by linear regression.

Test organism. *A. viridans* was cultured at 30° C for 24 hours in broth containing 200 ml yeast infusion per litre. The same medium served for the recovery of organisms subjected to heat tolerance testing. In order to imitate the original conditions, the bacteria were exposed to heat in ham infusion containing 1.1 per cent salt at pH 6.5.

For the determination of the D value, 5 tubes were inoculated for each exposure time. The most probable viable count per ml was estimated by probability calculations. The D value was calculated by use of the equation

$$D = \frac{U}{\log N - \log \bar{x}}$$

where U = time of exposure, N = viable count before exposure, $\bar{x}$ = viable count after exposure.

In calculating the needed exposure to heat, the industrial data concerning inside temperature changes during processing of canned ham, the D value for *A. viridans*, and the total microbial count of canned products prior to processing, were considered. As to the last value, SIMONFFY [5] found 10^7 organisms per g in such products. In the present experiments for safety reasons the maximum initial count was estimated at 10^{12} per g, which was to be decreased during processing to 10^2 per g. For a decrease of the viable count by 10 logarithmic cycles, the tenfold of the decimal reduction time ($10D$) is needed. In the calculations the reciprocal of this value, i.e. the rate of destruction, was used.

Results

The D values obtained in the experiments are presented in Table I.

By plotting the results on a graph, with the exception of data obtained at 70°C , the points fell along a straight line. As the deviation from the straight line between 65 and 70°C was probably due to experimental errors, this temperature range was omitted from the calculation of the equation.

Table I

D and log D values at various temperatures for A. viridans

Temperature $^\circ\text{C}$	D	$\log D$
55	19.86	1.2979
	22.37	1.3497
60	10.08	1.0035
	7.87	0.8960
	8.03	0.9047
	7.36	0.8669
	8.67	0.9380
	6.67	0.8241
65	2.68	0.4281
	2.42	0.3838
70	1.34	0.1271
	1.50	0.1761

The equation for the straight line was

$$y = 6.397 - 0.0918 x$$

In Fig. 1 the straight part of the graph is represented by a continuous, the part between 65 and 70°C by a dotted, line.

The 95 per cent confidence interval of the coefficient of x was $b = \pm 0.0191$. Thus the equation for the straight line could be expressed as

$$y = 6.397 - (0.0918 \pm 0.0191) x$$

In order to compare the results with data of other authors, the z value (slope) was also determined. The comparison of z and D values for various microorganisms is presented in Table II.

It is seen that the results obtained with our strain stand close to the highest z and D values, that is, *A. viridans* is one of the most heat resistant organisms among vegetative bacteria.

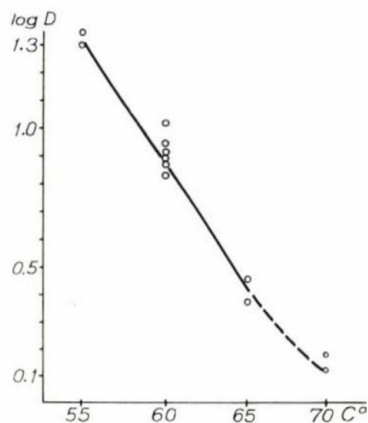


Fig. 1. Thermal death curve for *A. viridans*

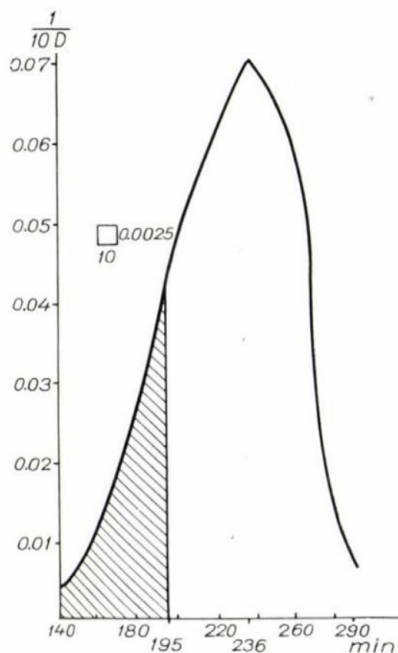


Fig. 2. Graphic calculation of heat-processing requirement. Weight of canned shoulder 4.34 kg; temperature of water bath, 86°C; time of exposure, 15 min.; then 75°C, 221 min.

Calculations for the necessary heat treatment were carried out by determining the unit area that fell under the special thermal death curve expressing destruction rate *vs.* time. The data needed for the calculations are summarized in Table III, the results are shown in Figs. 2, 3 and 4.

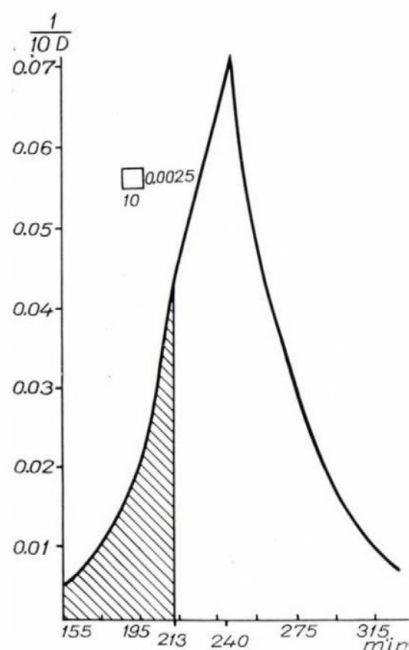


Fig. 3. Graphic calculation of heat-processing requirement. Weight of canned shoulder 3.9 kg; temperature of water bath, 78°C, time of exposure, 240 min.

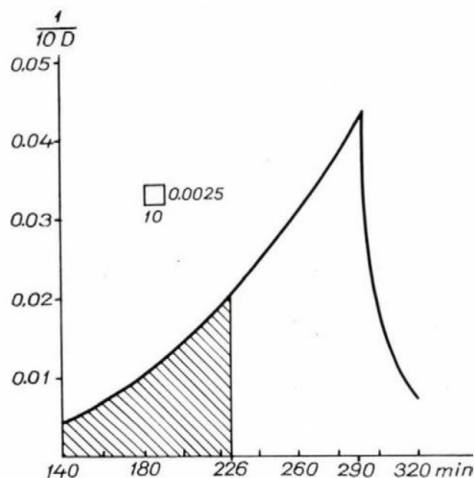


Fig. 4. Graphic calculation of heat-processing requirement. Weight of canned ham, 5.96 kg; temperature of water bath, 90°C, time of exposure, 25 min.; then 74°C, 265 min.

Table II
Comparison of z and D values for different microorganisms

Author	z value C°		D value min.			Organism
	with		A. viridans*	Other organism		
	non spore-forming	spore-forming				
GROSS and VINTON [6]	5					Staphylococcus
ANELLIS <i>et al.</i> [7]	4 — 6					Salmonella
SCHELHORN [8]	3 — 5					Yeast
SCHMIDT-LORENZ [9]	9.3—10.1					Salmonella
MØLLER-MADSEN <i>et al.</i>						
[10]	2.3		1.6	68°	3.5	<i>Str. faecalis</i>
	4.1		7.9	60°	0.33	Salmonella
	4.3		5.2	62°	0.33	Brucella
	4.4		26	54°	0.5	<i>Str. pyogenes</i>
	4.7		7.9	60°	0.5	Staphylococcus
KELLS and LEAR [11] ...	4.8— 5.2					<i>M. tuberc., bovis</i>
OTT <i>et al.</i> [12]	6.4		7.9	60°	13.5	<i>Str. faecalis</i>
			2.7	65°	1.9	<i>Str. faecalis</i>
			1.2	71°	0.33	<i>Str. faecalis</i>
WHITE [13]			7.9	60°	2—6	<i>Str. faecalis</i>
ANDERSON <i>et al.</i> [14]		11				<i>B. thermoacidurans</i>
KAPLAN <i>et al.</i> [15]		10				<i>Cl. sporogenes</i>
ESSELEN and PFLUG [16]	8.8—12					<i>Cl. sporogenes</i> (PA 3679)
SOGNEFEST <i>et al.</i> [17]	5.5— 7.7					<i>Cl. botulinum</i>
(at different pH)	or 8.8—12					and <i>sporogenes</i>
KNOCK <i>et al.</i> [18]	9					<i>B. thermoacidurans</i>
SECRIST and STUMBO [19]	9.8—10.4					<i>Cl. sporogenes</i> (PA 3679)
INCZE	10.89					<i>A. viridans</i>
	(9.02—					
	13.7)					
	P=95%					

* D values for *A. viridans* are presented as a comparison to the D values for other microorganisms at the corresponding temperatures

Thus, considering the heat-resistance of *A. viridans*, canned products in Hungary are subjected to a 2 to 3-fold overprocessing.

From the results it follows that within reasonable limits, the heat-processing of the products can be decreased without the danger of microbiological spoilage.

Table III

Calculated D values and destruction rates needed for the determination of heat-processing requirements

Temperature C°	D	16D	1/10 D
55	22.5	225	0.0044
56	18.8	188	0.0053
57	15.0	150	0.0066
58	12.0	120	0.0083
59	9.8	98	0.0102
60	7.9	79	0.0126
61	6.5	65	0.0154
62	5.2	52	0.0192
63	4.2	42	0.0238
64	3.4	34	0.0294
65	2.7	27	0.0370
66	2.2	22	0.0454
67	1.8	18	0.0555
68	1.6	16	0.0625
69	1.4	14	0.0714
70	1.3	13	0.0769
71	1.2	12	0.0833

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ACTINOMYCES FINLAYI N. SP.

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(Received April 18, 1963)

Summary. *Actinomyces finlayi* is a new species of *Actinomyces* (*Streptomyces*) section VII (SZABÓ and MARTON, 1962). It is morphologically and physiologically distinguishable from *Actinomyces acrimycini* GAUZE *et al.* 1957, *Streptomyces flaveolus* (WAKSMAN 1919) WAKSMAN *et* HENRICI 1948, *Sir. albogriseolus* BENEDICT *et al.* 1954 (*Griseoflavus* series) and from *Act. griseostramineus* GAUZE *et al.* 1957 (*Viridans* series). The type strain of *Act. finlayi* n. sp. (R-1-30) produces a characteristic green endopigment in the vegetative mycelium; therefore the strain has been compared with all available green *Actinomyces* type cultures. A key for the determination of green pigment-producing actinomycetes belonging to various series (*Flavovirens*, *Murinus*, *Viridans*, etc.) has been elaborated.

Some cultures of actinomycetes isolated from the A_{II} horizon of moder rendzina ("Mullartige Rendsina" [6]) in Sopron, West Hungary, have been identified as special types of green actinomycetes. In this paper, one of these cultures (R-1-30 = FBUA 1869), the holotype of a new species, is described and a key for the determination of actinomycetes producing green endopigment in the vegetative mycelium is presented.

Materials and methods

Carbon and nitrogen utilization spectra were examined on PRIDHAM and GOTTLIEB's synthetic medium [10]. Chromogenicity was tested on tyrosine-yeast extract agar as described by ETTLINGER *et al.* [2]. Production of H₂S and the melanin reaction were examined by the combined method of TRESNER and DANGA [14]. Classification of the strains into series was done by the system of SZABÓ and MARTON [13]. Cultural properties were examined by use of media referred to in the papers of LINDENBEIN [8] and SZABÓ and MARTON [13]. The classification of aerial mycelium, or, more exactly, of spores, according to colour groups, was performed by taking into consideration the proposition of ETTLINGER *et al.* [2]. Mutual antagonism was studied on CONN's agar modified by STAPP [11]. Over the Conn agar layer seeded with the antagonist and pre-cultivated for 5 days, another layer of nutrient agar containing the test organism was poured. The type strains used in this study were obtained from international culture collections (ATCC, CBS, INA, etc.).

Results

Systematic position of Act. finlayi n.sp. (strain R-1-30). Colour of spores: cinereus; type of sporophores: spira; colour of vegetative mycelium on certain media: flavus-brunus + viridis. The position of the strain in the *Viridans* "complementary series" of the *Griseoflavus* "main series" is determined by the combination of these three properties [13]. Among the sporo-

phores real closed and compact forms never occurred, most of them were of the rectus flexibilis type. Only the occasional occurrence of "short spirals" (with one or two exceptionally elongated curves) indicated that the sporophores should be included in the spira category. This finding was confirmed by Dr. R. HÜTTER [4], who also regarded strain R-1-30 as belonging to the spira section. Electronmicroscopic examination showed hairy formations on the surface of spores (Fig. 1). The melanin and Tresner-Danga reactions were negative. On the basis of these properties, strain R-1-30 stands most closely to *Act. acrimycini* GAUZE et al. 1957, *Str. flaveolus* (WAKSMAN 1919) WAKSMAN et HENRICI 1948, *Str. albogriseolus* BENEDICT et al. 1954 (Griseo-flavus series) and *Act. griseostramineus* GAUZE et al. 1957 (Viridans series). Due to their hairy spore surface, these species can sharply be differentiated from other members of the mentioned series.

Taxonomic differentiation of Act. finlayi n.sp. Comparative examinations were carried out with type cultures of the listed species. Our strain differed most definitely from the neomycin complex-producing strain NRRL B-1305 of *Str. albogriseolus*. (i) The cultural characteristics were entirely different (strain B-1305 is no green pigment-forming organism, its spores are not typically cinereus in colour, but are of a shade azureus, as observed also by ETTLINGER et al. [2]). (ii) Sporophores of B-1305 are regular closed spirals with monopodial branching. (iii) Between R-1-30 and B-1305 there is a definite mutual antagonism. (iv) Between the two strains there is a considerable physiological difference (B-1305 reduces nitrates to nitrites, R-1-30 fails to do so; the two cultures differ in antibiotic activity, antibiotic sensitivity and carbon utilization patterns, etc.).

Of the further 3 species only *Act. griseostramineus* produces green endopigment and belongs to the same series (Viridans) as R-1-30. However, the type culture of the former, INA N 10 381, in contrast to R-1-30, gives a positive melanin and Tresner-Danga reaction and definitely utilizes d-mannitol and d-fructose as carbon sources (Table I). INA N 10 381 strongly inhibits the growth of R-1-30 (Table II). The antibiotic activity pattern of the two strains also differs considerably. Our strain is not identical with *Str. flaveolus* ATCC 3319 and *Act. acrimycini* INA N 7699. The latter strains exhibit different carbon source utilization spectra (Table I), produce no green endopigment and differ from R-1-30 in numerous cultural and physiological properties (see the diagnosis of R-1-30). It should be noted that *Str. flaveolus* ATCC 3319, which is the type strain for *Str. flaveolus* (WAKSMAN) sensu ETTLINGER et al. 1958 [2], and is characterized by the combination cinereus-spira-hairy spore surface-achromogenicity, exerts an uncommonly powerful antibiotic action against R-1-30 (Table II). Therefore, as strain R-1-30 differs in many differential diagnostically important properties from the most closely related species, its recognition as a new species is justifiable. In Tables

I and II the behaviour of some other, green pigment-producing Actinomyces and Streptomyces species is also presented.

Table I

Carbon source utilization, melanin and H_2S production by type strains of some Actinomyces species

	<i>Str. viridis</i> ATCC 3372	<i>Act. atroolivaceus</i> INA N 1580/53	<i>Str. flavolus</i> ATCC 3319	<i>Act. mutabilis</i> INA N 472	<i>Act. malachiticus</i> INA N 399/55	<i>Act. finlayi</i> R-1-30	<i>Act. actinomyces</i> INA N 7699	<i>Act. griseostramineus</i> INA N 10381
Maltose	+++	++	+++	+++	+++	++	+++	+++
Raffinose	—	—	+	—	++	—	—	—
Rhamnose	+++	++	+++	+++	+++	++	+++	+++
d-Xylose	+++	++	++	+++	+++	+++	+++	+++
d-Mannitol	++	+++	+++	+++	+++	—	+++	+++
d-Arabinose	—	+	+++	+++	+++	++	+	+++
d-Fructose	+++	++	+++	+++	+++	—	+++	+++
Dextrin	+++	+++	+++	+++	+++	+++	+++	++
Inulin	—	—	+	—	—	—	—	—
Control without C	—	—	—	—	—	—	—	—
Melanin reaction	—	—	—	—	—	—	—	+
Tresner—Danga reaction	—	—	—	—	—	—	—	+++
Starch	+++	+++	+++	+++	+++	+++	+++	++

— = No growth or negative reaction; + = utilization or reaction doubtful; ++ or +++ definite utilization or positive reaction

Diagnosis of *Act. finlayi* n.sp. Named in honour of A. C. FINLAY, discoverer of oxytetracycline. **Type strain:** R-1-30 (FBUA 1869). **Morphology:** The vegetative mycelium measures 0.5–0.8 μ in diameter. Chlamidospores are not formed. Generally rectus flexibilis sporophores, with some elongated spirals consisting of 1 or 2 curves. The spores are covered with fine hairy formations longer than 1 μ . Aerial mycelium pulverulentus. **Cultural behaviour:** Glucose-asparagine agar: aerial mycelium (AM) ash grey, substrate mycelium (SM) colourless, soluble pigment (SP) brownish-yellow. Glucose agar: AM nil, SM greenish-yellow, SP yellow. Potato: AM nil, SM green, SP nil. Synthetic agar: AM grey, SM yellowish-green, SP yellow. Oatmeal agar: AM grey, SM green, SP nil. Glycerol-glycine agar: AM nil, SM green, SP nil. Starch agar: AM nil, SM green, SP nil. **Classification:** Viridans series (Combination: SM flavus-brunus + viridis; AM cinereus; Sporophore, spira). **Physiological behaviour:** Nitrites are not produced from nitrates. Milk is coagulated and peptonized. Paraffin and wax are not utilized. Gelatin is powerfully liquefied.

Table II

Sensitivity and antagonistic action of *Act. finlayi* n.sp. against *Streptomyces* and *Actinomyces* strains

Tested on Conn agar modified by Stapp

Antagonistic activity of various actinomycetes against strain R-1-30. Radius of inhibition zone, mm													
9	12	20	40	6	3	19	9	12	20	11	3	0	12
<i>Act. acrimycini</i> INA N 7699	<i>Str. albobogrusculus</i> NRRL B-1305	<i>Act. atroolivaceus</i> INA N 1580/53	<i>Str. flavoculus</i> ATCC 3319	<i>Str. flavovirens</i> IMRU 3320	<i>Str. flavovirens</i> IPV 169x	<i>Act. griseostramineus</i> INA N 10381	<i>Act. maluchiticus</i> INA N 399/55	<i>Act. mutabilis</i> INA N 472	<i>Str. olivaceus</i> ATCC 3335	<i>Act. roseoviridis</i> INA N 3617	<i>Str. sp. (Murinus)</i> S-40	<i>Str. viridis</i> ATCC 3372	<i>Str. viridochromogenes</i> NRRL B-1227
0	4	11	2	0	0	0	8	6	0	2	2	0	3
Antagonistic activity of strain R-1-30 against various actinomycetes. Radius of inhibition zone, mm													

Shows poor hemolytic and amylolytic activity. *Antibiotic activity*: ineffective against *E. coli*, *B. subtilis*, *S. marcescens*, *Rh. meliloti*, *Staph. aureus*, *Sacch. carlsbergiensis*, *Aspergillus niger*, *Trichothecium roseum*; slight activity against *Sarcina lutea* and some actinomycetes. Grows profusely at 36°C; no growth at 40°C. On synthetic Pridham-Gottlieb medium utilizes as carbon sources d-galactose, l-arabinose, rhamnose, d-xylose, d-cellobiose, maltose, glycerol, glycogen, dextrin, starch, sodium citrate. D-fructose, d-mannitol and sodium oxalate are not utilized. Grows poorly or not at all in the presence of urea, l-cystine, dl-methionine or nucleic acid as sole sources of nitrogen. Other nitrogen sources as dl-alanine, glycine, dl-serine, dl-threonine, dl-valine, cysteine, dl-tryptophan, l-glutamic acid, dl-aspartic acid, l-arginine, l-histidine and peptone are rapidly utilized. On bromocresol purple agar produces acid from maltose and mannose but not from lactose or xylose.

Key for the determination of "green actinomycetes". By comparing the most important green substrate mycelium-producing type strains of the genus *Actinomyces* syn. *Streptomyces*, the following key has been prepared. The key may be of aid in the routine determination of these organisms.

- 1a Colour of spores azureus ... *Str. viridochromogenes* (KRAINSKY 1914)
WAKSMAN et HENRICI 1948. Strain NRRL B-1227
- 1b Colour of spores other 2
- 2a Colour of spores cinnamoneus 3

2b	Colour of spores cinereus	4
3a	Sporophore spira ... <i>Str. sp.</i> (Murinus series) SZABÓ <i>et al.</i> 1962. Strain S-40.	
3b	Sporophore rectus flexibilis ... <i>Act. roseoviridis</i> GAUZE <i>et al.</i> 1957. Strain INA N 3617.	
4a	Sporophore rectus flexibilis	5
4b	Sporophore spira	9
5a	Melanin reaction positive ... <i>Str. herbaricolor</i> KAWATO <i>et</i> SHINOBU 1959. Strain 608.	
5b	Melanin reaction negative	6
6a	Raffinose positive	7
6b	Raffinose negative	8
7a	Arabinose and rhamnose positive ... <i>Str. flavovirens</i> (WAKSMAN 1919) WAKSMAN <i>et</i> HENRICI 1949. Strain IMRU 3320.	
7b	Arabinose and rhamnose negative ... <i>Act. olivoviridis</i> KUTSHAEVA <i>et al.</i> 1960. Strain 2718.	
8a	Lactose and sorbitol positive ... <i>Str. olivaceus</i> (WAKSMAN 1919) WAKSMAN <i>et</i> HENRICI 1948. Strain CBS Waksman.	
8b	Lactose and sorbitol negative ... <i>Act. fulvoviridis</i> KUTSHAEVA <i>et al.</i> 1960. Strain 143-X.	
9a	Melanin reaction positive ... <i>Act. griseostramineus</i> GAUZE <i>et al.</i> 1957. Strain INA N 10 381.	
9b	Melanin reaction negative	10
10a	Raffinose positive	11
10b	Raffinose negative	12
11a	Spore surface hairy ... <i>Str. flaveolus</i> (WAKSMAN 1919) WAKSMAN <i>et</i> HENRICI 1948. Strain IMRU 3319.	
11b	Spore surface spiny ... <i>Act. malachiticus</i> GAUZE <i>et al.</i> 1957. Strain INA N 399/55.	
12a	Mannitol and fructose negative ... <i>Act. finlayi n.sp.</i> Strain R-1-30.	
12b	Mannitol and fructose positive	13
13a	Spore surface hairy ... <i>Act. acrimycini</i> GAUZE <i>et al.</i> 1957. Strain INA N 7699.	
13b	Spore surface not hairy	14
14a	Spore surface warty, starch hydrolysis negative. <i>Act. atroolivaceus</i> GAUZE <i>et al.</i> 1957. Strain INA N 1580/53.	
14b	Spore surface smooth, starch hydrolysis positive ... <i>Act. mutabilis</i> GAUZE <i>et al.</i> 1957. Strain INA N 472.	

Note. In the key *Act. mutabilis* is designated as a melanin negative organism, although from the paper of GAUZE *et al.* [3] it seems to be melanin positive. In the present investigations strain INA N 472 was definitely Tresner—Danga

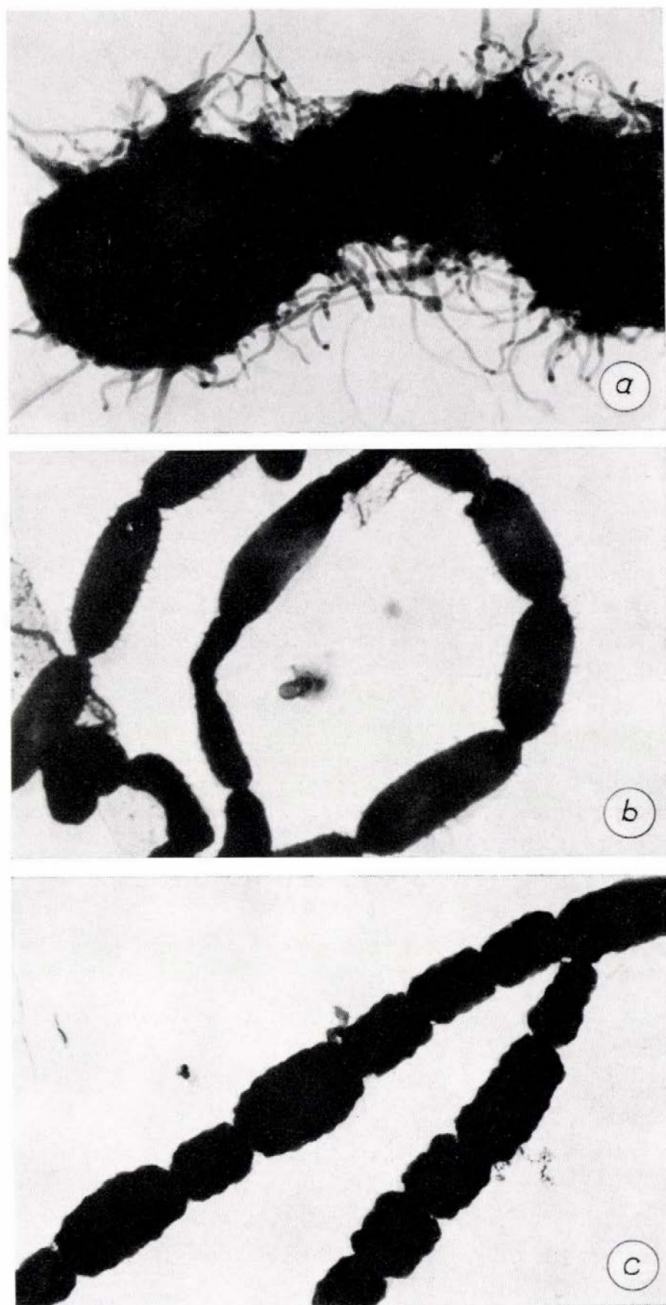


Fig. 1. Electronmicrographs showing the spore surface morphology of some Actinomyces type strains

- (a) *Act. finlayi* n.sp. Strain R-1-30. $\times 8000$
- (b) *Act. malachiticus*. Strain INA N 399/55. $\times 4450$
- (c) *Act. atroolivaceus*. Strain INA N 1580/53. $\times 4450$

and melanin negative. According to PREOBRAZHENSKAIA *et al.* [9], various strains of *Act. malachiticus* differ in spore surface. The spore surface of the available INA N 399/55 strain was regularly spiny.

No data were found in the literature as to the spore surface of *Act. atroolivaceus*. The spores of strain INA N 1580/53 (Fig. 1) were similarly of the characteristic warty surface as that observed by TRESNER *et al.* [15] in *Str. olivaceus* strain ATCC 12 019. Being very closely related to these organisms, strains *flaveolus* IMRU 3319 (ATTC 3319) and *acrimycini* INA N 7699, although they do not belong to the strictly defined green actinomycetes, were included in the key. Moreover, some authors consider that certain real green Actinomyces strains may be identical with species *flaveolus* or *acrimycini*.

Discussion

The determination and differentiation of *Act. finlayi* n.sp. from the more closely related organisms is easy if the characteristic spore surface morphology is considered. Spores covered with hairy formations are produced only by some occasional species, which, with the exception of *Str. prasinopilosus*, ETLINGER *et al.* 1958., on the basis of other morphological and physiological properties, can be regarded as belonging to a group of common origin. *Act. finlayi* n.sp. could sharply be differentiated from other members of this group.

Some years ago the systematic importance of mutual antagonism was a much debated problem. KRASSILNIKOV *et al.* [7], persisting in their original conception, consider the antibiotic activity and sensitivity spectra as one of the most important criterion of differentiating among various species. Recently, WAKSMAN himself [16] has based the differentiation of *Str. griseus* WAKSMAN *et* HENRICI 1948 and *Str. griseinus* WAKSMAN 1959 on the finding that strains within one of these species are not antagonistic to each other, but are able to inhibit the growth of strains belonging to the other species and *vice versa*.

In our opinion antibiotic activity is not so characteristic as the sensitivity spectrum. *E.g.* the streptomycin production by various *Str. griseus* strains may vary as far as complete inactivity, but most of the inactive strains retain their streptomycin resistance. It is remarkable that *Act. finlayi* n.sp. strain R—1—30 is powerfully, or even extremely antagonized by type strains of the related species.

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MYCOBACTERIUM MINETTI (PENSO ET AL. 1952) BACTERIOLOGICAL AND EPIDEMIOLOGICAL OBSERVATIONS

By

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Summary. From human material 21 *M. minetti* (PENSO *et al.* 1952) strains have been isolated. The cultures were identified by considering their morphological, biochemical, cytochemical properties, pathogenicity to animals and phage sensitivity. On the basis of its behaviour, *M. minetti* should be regarded as a separate species belonging to saprophytic mycobacteria. Its human pathogenicity could not be verified. It is assumed that the organism, similarly to soil bacteria, is capable of demineralization.

The taxonomic position, pathogenicity and epidemiology of atypical (anonymous) mycobacteria have extensively been studied [1, 2]. Although recently numerous new characteristics of these organisms have been recognized, their systematic position is still undecided. RUNYON's generally used classification is not satisfactory. According to HAUDUROY the aim should be the exact microbiological determination and grouping of these bacteria [3].

The present paper deals with an organism included by RUNYON in group 4, the most characteristic member of which is *M. fortuitum*. Identification of our group 4 strains collected for years in this institute, revealed some well defined strains among the cultures. On more detailed examination these cultures turned out to be *M. minetti* (PENSO *et al.* 1952).

From osteopathic lesions and mesenterial lymph glands of cattle MINETT [4] isolated four similar Mycobacterium strains. The organisms were described as *M. avium*.

PENSO *et al.* [5] in 1952 studied the morphological and biological properties of the four strains; neither of the cultures corresponded to *M. avium*. On the basis of their characteristic behaviour, the strains were included in a new species, which, in honour of MINETT, was named *M. minetti*. The same authors [5a] examined also *M. fortuitum*, Cruz (nomen rejecendum), GORDON's *M. fortuitum* and DARZIN's *M. giae*. They noted that some international type strains were insufficiently and even erroneously characterized, and, on the other hand, that *M. fortuitum* and *giae* were very similar to *M. minetti*.

Materials and methods

Presumably saprophytic cultures isolated from routine material, were subjected to phage sensitivity testing. Thus 21 strains lysed exclusively by *M. minetti* phage were selected. The morphological and biochemical examinations listed below were performed with these

Table I
Properties of

1	2	3	4	5				6									
Serial No.	Designation No.	Origin	Sahyyn medium	10 day growth				Boenicke's acylamidases									
				25°C		37°C		Acetamide	Allantoin	Benzamide	Urea	i-Nicotinamide	Malonamide	Nicotinamide	Pyrazinamide	Salicylamide	Succinamide
				Sula	Loew.	Sula	Loew.										
of strains																	
1	24	Gastric juice	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-
2	25	Sputum	+	+	+	+	+	+	±	-	+	-	-	-	-	-	-
3	26	Liquor	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-
4	29	Sputum	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
5	31	Sputum	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
6	33	Sputum	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-
7	41	Sputum	+	+	+	+	+	-	+	-	++	-	-	-	-	-	-
8	45	Sputum	+	+	+	+	+	-	-	-	±	-	-	-	-	-	-
9	49	Sputum	+	+	+	+	+	-	+	-	++	-	-	-	-	-	-
10	50	Urine	+	+	+	+	+	±	-	-	+	-	-	-	-	-	-
11	52	Sputum	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-
12	54	Sputum	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-
13	56	Sputum	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-
14	62	Sputum	+	+	+	+	+	+	+	-	++	-	-	-	-	-	-
15	67	Sputum	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-
16	77	Sputum	+	+	+	+	+	±	+	-	++	-	-	-	-	-	-
17	82	Sputum	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-
18	95	Sputum	+	+	+	+	+	-	+	-	+	-	-	-	-	-	-
19	99	Sputum	+	+	+	+	+	+	+	-	++	-	-	-	-	-	-
20	217	Laryngeal swab	+	+	+	+	+	+	±	-	+	-	-	-	-	-	-
21	219	Laryngeal swab	+	+	+	+	+	-	-	-	++	-	-	-	-	-	-
M. mini-netti-	Roma	PENSO et al.	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-

cultures. As a control, PENSO's *M. minetti* strain was used. The results have been so tabulated that in Table I column 1 indicates the serial number, column 2 the designation number and column 3 the origin of the cultures.

Growth on Sahyyn medium [18]. (Column 4).

Growth in fluid Sula medium modified by us and on Loewenstein-Jensen medium, was tested at room temperature and at 37° C after 10 days incubation (Column 5).

B: alpha-naphthylamine, 0.3 g; distilled water, 70 ml; after filtration, 30 ml glacial acetic acid.) Before use 1 part solution "A" was added to 1 part solution "B", then to each tube containing 8 ml medium 1 ml of the mixture was pipetted. The tubes were left to stand for 10 minutes before reading.

Tests for iron metabolism, as worked out in this laboratory, were carried out in three different ways.

(a) In the modified Sula or Loewenstein medium iron ammonium citrate was incorporated at 2 per cent concentration. When incubated for 10 days on these media, cultures capable of accumulating iron produced red growth.

(b) In order to examine the ability of the culture to gain energy from iron compounds, in Sahuyn's medium the carbon source (glucose) was substituted for ferrous sulphate, ferric ammonium citrate or ferric chloride. The medium was inoculated with 0.02 mg of a bacterial suspension washed in saline three times. Growth was examined under aerobic conditions.

(c) Iron in the bacterial cells was demonstrated by means of a micro-reaction. The culture grown on the iron-containing medium was washed three times then smeared onto a slide. The smear was treated with $NHCl$, then with potassium ferri- or ferrocyanide. The presence of iron was indicated by a Berlin blue or Turnbull blue colour, respectively. The reaction was checked under the microscope.

Sensitivity to isonicotinic acid hydrazide, streptomycin, and *p*-aminosalicylic acid was tested in the modified Sula medium at drug concentrations shown in Column 10 of Table I. The tubes were inoculated with 0.02 mg bacteria (wet weight) and incubated for 4 weeks.

Catalase reaction (Column 11) was performed qualitatively.

Utilization of hydrocarbon sources (Column 12).

The utilization as carbon source of gasoline and paraffin was tested as follows. To 7 ml Sahuyn medium devoid of glucose 1 ml gasoline or liquid paraffin were added. The cells were washed three times in saline then to each tube 0.02 mg bacteria were added. Incubation lasted for 3 weeks. It was characteristic that some strains grew only in the form of a layer between the gasoline and the medium. In the medium with liquid paraffin, growth was characterized by whitish-grey pellicles formed on the surface of oil globules.

Mouse pathogenicity (Column 13). PENSO [5] described that mice inoculated intravenously with *M. minetti* produced characteristic rolling movements occurring persistently in the same direction. Each animal was given a dose containing 1 mg cells (wet weight). Observation lasted for 2 weeks.

Phage sensitivity (Table II). Phages for *M. phlei*, *pellegrino*, *minetti*, *smegmatis*, *butyricum*, *friburgensis*, *rabinowitsch* were used. The phages were isolated in our institute [10], by inoculation of soil samples with the corresponding bacterial strain. In addition, phages Polanus, MyF₁ P/58, MyF₂ P/59, MyF₃ P/59 obtained from Prague, and D29 (FROMAN) obtained from U.S.A. were employed. Each phage suspension contained 10^6 particles per 1 ml.

Phage-typing was carried out by pipetting into 3 ml of semisolid agar 10 drops of the homogenized 48 hour liquid culture of the strain. After mixing thoroughly, the culture was layered over the surface of an agar plate. The plates were left to stand for one hour, then with test tubes wells were bored into the agar layer. The different phage suspensions were pipetted into the wells. The plates were then left to dry for another hour and were subsequently transferred to a 37°C incubator. Readings were made after 48 hours. The phage activity extended over an area 8–10 mm in diameter, showing plaque-like, confluent or total lysis.

Results

Considering the growth in Sahuyn medium, the control culture and our strains uniformly belonged to the heterotrophic group I, the members of which utilize inorganic ammonium salts as a sole source of nitrogen. In a previous paper it was shown that under certain conditions that medium is useful for the differentiation of obligatory parasitic and facultative pathogenic mycobacteria classified into the group of saprophytic mycobacteria.

No difference was revealed between the control strain and the isolated cultures as to growth at 25° and 37°C. Good growth at 25°C indicates that the culture is no obligatory human parasite, as it is able to multiply at varying temperatures.

Table II
Phage sensitivity of M. minetti

Serial no.	Designation no.	Phage											
of strains		phlei	pellegrini	minetti	smegmatis	butyricum	friburgensis	rabino-witsch	polanus	D 29 (Froman)	MyF ₁ P/58	MyF ₂ P/59	MyF ₃ P/59
1	24	—	—	+	—	—	—	—	—	—	—	—	—
2	25	—	—	+	—	—	—	—	—	—	—	—	—
3	26	—	—	+	—	—	—	—	—	—	—	—	—
4	29	—	—	+	—	—	—	—	—	—	—	—	—
5	31	—	—	+	—	—	—	—	—	—	—	—	—
6	33	—	—	+	—	—	—	—	—	—	—	—	—
7	41	—	—	+	—	—	—	—	—	—	—	—	—
8	45	—	—	+	—	—	—	—	—	—	—	—	—
9	50	—	—	+	—	—	—	—	—	—	—	—	—
10	52	—	—	+	—	—	—	—	—	—	—	—	—
11	54	—	—	+	—	—	—	—	—	—	—	—	—
12	56	—	—	+	—	—	—	—	—	—	—	—	—
13	62	—	—	+	—	—	—	—	—	—	—	—	—
14	67	—	—	+	—	—	—	—	—	—	—	—	—
15	77	—	—	+	—	—	—	—	—	—	—	—	—
16	82	—	—	+	—	—	—	—	—	—	—	—	—
17	95	—	—	+	—	—	—	—	—	—	—	—	—
18	49	—	—	+	—	—	—	—	—	—	—	—	—
19	99	—	—	+	—	—	—	—	—	—	—	—	—
20	217	—	—	+	—	—	—	—	—	—	—	—	—
21	219	—	—	+	—	—	—	—	—	—	—	—	—
<i>M. minetti</i>	Roma	—	—	+	—	—	—	—	—	—	—	—	—

Of BOENICKE's amides, urea was utilized by all isolated strains as well as by the control strains. Most strains also utilized acetamide and allantoin. As characteristic of the behaviour of *M. minetti*, with other amides uniformly negative reactions were obtained. As compared to PENSO's results [5a], some reactions were discrepant *e.g.* those with allantoin. The discrepancy may have been due to the fact that we recorded only 4-hour reactions, while BOENICKE recommended that readings should be made also after 6 to 8 hours.

The nicotinic acid test was negative with all the examined strains. This is an important difference from the behaviour of obligatory parasitic mycobacterial strains.

Table III
Origin of M. minetti strains

Serial No. of strains	Designation No.	Patient	Clinical diagnosis	<i>M. tuberculosis</i>		Note
				Positive	Negative	
1	24	G.I.	Pulmonary tbc.		—	
2	25	H.K.	Pulmonary tbc.		—	
3	26	H.J.	Tbc. meningitis		—	*
4	29	H.B.	?			**
5	31	H.G.	Pulmonary tbc.	+		
6	33	K.K.	Non-specific pneumonia ..		—	
7	41	K.A.	Pulmonary tbc.		—	
8	45	L.L.	Pulmonary tbc.		—	
9	49	M.F.	Pulmonary tbc.	+		
10	50	M.E.	?			**
11	52	M.G.	Pulmonary tbc.	+		
12	54	N.J.	?			**
13	56	P.J.	Pulmonary tbc.	+		
14	62	S.E.	Pulmonary tbc.		—	
15	67	S.F.	Pulmonary tbc.	+		
16	77	T.M.	Pulmonary tbc.	+		
17	82	V.I.	?			**
18	95	M.R.	Pulmonary tbc.	+		
19	99	T.L.	Pulmonary tbc.	+		
20	217	K.G.	Pulmonary tbc.		—	
21	219	J.G.	Tbc. exsudative pleurisy ..	+		

* Post-therapeutic control ** Deficient data

The nitrite producing capacity of the strains was characteristic. All the 21 strains exhibited but a moderate nitrate reductase activity. Instead of VIRTANEN's [12], the Griess—Ilosvay reagent was applied because it allowed some quantitative estimation. Repeated testing under the same conditions always gave an orange colour. Our observations indicate that the quantitative nitrite reaction is characteristic of different mycobacterial strains.

Iron salts incorporated in liquid or solid media were taken up by the examined bacteria (Fig. 1). These substances were shown in the cells by a spot test (Fig. 2). However, the organisms were unable to utilize iron compounds as a source of energy, and thus, on iron-containing media devoid of carbon source, no growth occurred. A similar behaviour of numerous soil bacteria is an important feature in their differentiation from the iron bacteria [13].

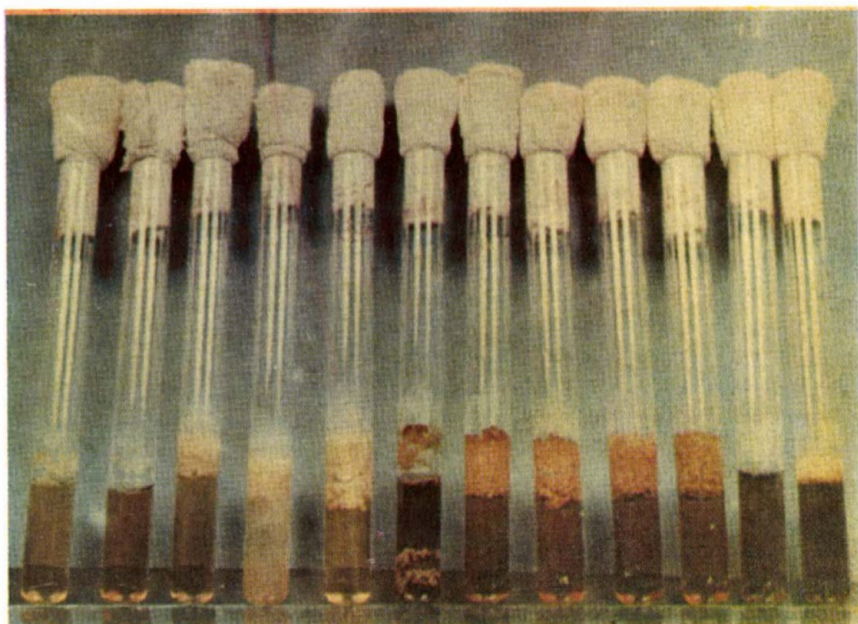


Fig. 1. Iron accumulation by *M. minetti*. From left to right: *M. tuberculosis* H37 Rv, Ravenel, avian S. jar., avian strain Mg., saprophytic unnamed strain 335, *M. minetti* PENSO, *M. minetti* 29, *M. minetti* 31, *M. minetti* 26, *M. minetti* 33, two *M. tuberculosis* strains from routine material

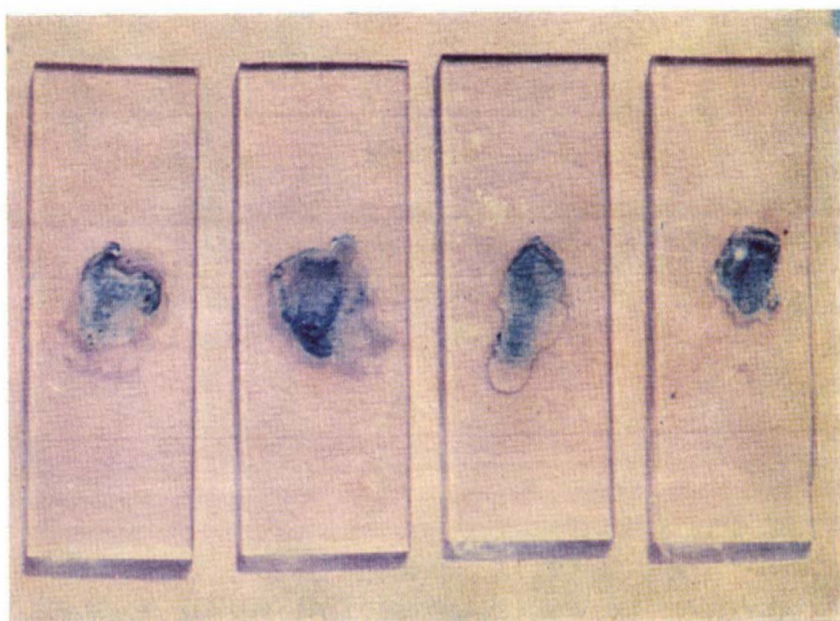


Fig. 2. Microreaction for the detection of intracellularly accumulated iron. Four saprophytic strains. 1 : 1. Negative reaction is colourless

A high degree of resistance to anti-tuberculous drugs is to a certain extent characteristic of saprophytic mycobacteria. Insusceptibility to INH and PAS is particularly common. Streptomycin resistance is not so definite, as sensitive strains may occur. Our *M. minetti* strains corresponded to the above susceptibility pattern.

In mouse pathogenicity experiments all living strains, when given intravenously, produced the characteristic rolling reaction. Non-living suspensions or cell lysates never caused such symptoms. It seems likely that some of the metabolic products of *M. minetti* are injurious to the central nervous system or to the eighth cranial nerve.

The catalase reaction was positive with all strains. None of the cultures utilized hydrocarbons as carbon source. Thus they differed from several *Nocardia* strains and some saprophytic mycobacteria genetically closely related to nocardiae (e.g. *M. scrofulaceum*).

The *M. minetti* phage isolated in this laboratory lysed all strains that were diagnosed as *M. minetti*. Generally, confluent or total lysis was observed.

Source of cultures. Each strain was isolated from patients treated in various institutions situated in different parts of the country. In most cases tuberculosis was verified by multiple cultural examinations. In bacteriologically negative cases the clinical diagnosis undoubtedly revealed the tuberculous aetiology. In four cases the clinical data were deficient; one patient had been admitted with non-specific pneumonia. The relevant data are presented in Table III.

Discussion

The growth in Sahuyn medium and at room temperature, the behaviour to Boenicke's amide compounds and the nicotinic acid reaction uniformly indicate that *M. minetti* does not belong to the obligatory parasitic mycobacteria.

Our investigations have shown that the obligatorily parasitic human and bovine *M. tuberculosis* strains do not grow in Sahuyn medium and at room temperature. Of Boenicke's compounds they usually decompose urea; the reaction with the other amides is variable or unstable. The nicotinic acid test is positive with human and variable with bovine strains, and invariably negative with saprophytic mycobacteria.

Reduction of nitrates is a common property of soil bacteria. Numerous isolations of phages and bacteria have shown that a considerable proportion of saprophytic mycobacteria is permanently present in soil. Demineralization is partly due to soil microorganisms. Of mycobacteria, only *M. balnei* is unable to produce nitrites. Nitrates are reduced by many facultative pathogenic organisms encountered in medical bacteriology. Among the mycobacteria, *M. phlei*, *pellegrino*, *kansasii* (Hauduroy) powerfully produce nitrites. *M. minetti*, as shown by the present investigations, exerts a medium degree of

nitrate-reducing capacity. The non-nitrite-producing *M. balnei* occurs in the water of baths and in sewage, but not in soil. Although strains within one and the same species may somewhat differ as regards nitrite production, this property is very characteristic, and may be adequately used for classification purposes.

Our investigations into the iron metabolism of mycobacteria revealed that saprophytic strains unlike parasitic ones, are able to accumulate iron compounds. Under aerobic conditions neither the former, nor the latter group of mycobacteria can utilize these substances as a source of energy. As to iron metabolism, *M. minetti* behaves similarly to saprophytic strains.

Considering its moderate sensitivity to streptomycin and its resistance to INH and PAS, *M. minetti* should be classified as a saprophytic species. As noted in a previous paper, variations in drug resistance are often encountered [11].

The characteristic rolling symptoms in the mouse are produced only by the intravenous injection of living organisms. No symptoms occur after the inoculation of dead bacteria or bacterial lysates. In our opinion, the presence of an exotoxin in *M. minetti* cultures may be responsible for the central nervous lesion. The problem awaits further investigation.

Some authors (PRISSICK and MASSON [14]) described a *Mycobacterium* strain (*M. scrophulaceum*) able to utilize hydrocarbons. We also succeeded in isolating a *M. scrophulaceum* strain from the cervical lymph gland of an eight-year-old child. *M. minetti*, similarly to most species, did not grow in paraffin or gasoline media. Of the phages isolated in this laboratory, the examined strains were lysed only by that specific for *M. minetti*. During isolation our phages came into contact only with the homologous mycobacterial strains; serological examinations in progress also indicate the homogeneity of *M. minetti* phage.

Accordingly, we regard phage-typing as an adequate screening test for *M. minetti*. This has been confirmed by the fact that our 21 strains behaved uniformly as to morphological, and biological properties considered necessary for final diagnosis. For a preliminary routine examination, rapid phage sensitivity testing is most advantageous.

Studies of the antigenic structure have revealed the specificity of certain antigens for *M. minetti* [15].

In the literature, mainly *M. minetti* strains isolated from soil or animals have been described. In the collection of PENSO *et al.* [5a] only 6 out of 63 strains originated from humans. FROMAN [16] described only one strain that caused infection in man. As to our 21 *M. minetti* strains, none of them could be made responsible for human disease. According to HAUDUROY [17], for estimating the pathogenicity of a given strain, the following criteria should be considered. (i) Was or was not the patient culturally positive for *M. tubercu-*

losis when harbouring the incriminated culture? (ii) Could tuberculosis be excluded clinically? (iii) Was the strain of supposed pathogenicity isolated on one or more occasions? Growth occurred in small or large numbers of colonies?

From the present examinations the mode of spread of *M. minetti* from man to man or from animals to man cannot be estimated. These problems, as well as those of human pathogenicity, need further investigations.

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BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

I. UNTERSUCHUNG DER ROLLE DER ESCHERICHIA COLI STÄMME

Von

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Zusammenfassung. Die Verfasser untersuchten im Laufe ihren einjährigen Beobachtungen die fermentativen Eigenheiten, das "O" Antigen, die Antibiotikaempfindlichkeit, die enteropathogene Rolle der aus den Stuhlproben von 253 an Enterocolitis leidenden und 349 gesunden Säuglingen bzw. Kleinkindern stammenden 967 *E. coli* Stämme sowie den Krankheitsverlauf der enteralen Kranken und arbeiteten die Daten derselben auf.

Laut den parallel mit den klinischen Beobachtungen durchgeführten bakteriologischen Untersuchungen kann die Coli-Ätiologie der Darmkatarrhe der Säuglinge im 1–6. Lebensmonat als wahrscheinlich betrachtet werden und es wird auf die fakultative enteropathogene Rolle der bei den Gastroenterocolitiden des Säuglingsalters im Dickdarmtrakt periodisch vorkommenden *E. coli* Stämme vom sogenannten »Übergangscharakter« hingewiesen.

Die Darminfektionen bilden ein auch aus praktischem Standpunkt wichtiges Fachgebiet der Säuglings- und Kinderheilkunde. In der ganzen Welt wird ihnen besondere Aufmerksamkeit gewidmet, nach Angaben der klinischen Statistiken bilden ja eben die enteralen Krankheitsprozesse einen bedeutenden Teil der akuten Infektionen des Säuglings- und Kindesalters. Hand in Hand mit der Entwicklung der bakteriologischen Diagnostik wird die Züchtbarkeit der bekannten enteropathogenen Stämme (*Shigella*, *Salmonella*, *E. coli*) ständig erfolgreicher und auch die präventive und therapeutische Bekämpfung dieser Infektionen wird immer wirkungsvoller. Dessenungeachtet sind bloß 45–50% der infektiösen Dyspepsien des Säuglings- und Kindesalters bakteriologisch verifizierbar.

Der hohe Prozentsatz der Magen- und Darmkatarrhe nicht eruierten Ursprungs veranlaßte uns, durch systematische Untersuchungen Angaben in bezug auf die kausale Rolle der im Darmtrakt am häufigsten vorkommenden Bakteriengruppen zu sammeln. Wir waren bestrebt, die ätiologische Rolle der *E. coli*, *Klebsiella*, *P. mirabilis*, *P. vulgaris*, *P. morganii*, *Ps. aeruginosa* und *Staphylococcus aureus* Gruppen zu klären, mit Vorbehalt der Möglichkeit einer eventuellen, durch Protozoone, Pilze oder Viren verursachten Darminfektion, worauf sich unsere Beobachtungen — abgesehen von einigen Ausnahmen — nicht erstreckten.

Wir untersuchten im Kinderspital in der Periode vom Januar 1958 bis zum Januar 1959 1204 Kranke. Bei 527 derselben konnten wir enterale Symp-

tome beobachten, während 677 sich als enteral symptomfrei erwiesen. Aus dem Stuhl der enteralen Kranken ließen sich bekannte enteropathogene Keime in 255 Fällen (48,3%), aus den Stuhlproben der enteral symptomfreien Kranken solche in 47 Fällen (6,9%) isolieren (Tabelle I). Aus dem Stuhl der an Enterocolitis Erkrankten konnte in 193 Fällen (36,6%) *E. coli dyspepsiae*, in 55 Fällen (10,4%) *Shigella*, in 6 Fällen (1,1%) *Salmonella* gezüchtet und in einem Fall (0,2%) *Giardia lamblia* gefunden werden. In den Stühlen der 47 symptomfreien Bakterienausscheider fanden wir verschiedene Serotypen von *E. coli*. Die serologische Verteilung der bekannten enteropathogenen Keime ist ersichtlich in Tabelle I.

Tabelle I

Stammtypen	Von enteralen Kranken	Von symptomfreien Ausscheidern
<i>E. coli</i> O : 111 B : 4	156	38
<i>E. coli</i> O : 55 B : 5	16	3
<i>E. coli</i> O : 26 B : 6	8	1
<i>E. coli</i> O : 86 B : 7	10	5
<i>E. coli</i> O : 112 B : 13	2	—
<i>E. coli</i> O : 2 B : 1	1	—
<i>Citrobacter</i> (mit Paratyphus »0« Antigen)	1	—
<i>Sh. flexneri</i>	16	—
<i>Sh. sonnei</i>	38	—
<i>Sh. boydi</i>	1	—
<i>S. enteritidis</i>	2	—
<i>S. paratyphi A</i>	1	—
<i>S. saint-paul</i>	1	—
<i>S. heidelberg</i>	1	—
<i>Giardia lamblia</i>	1	—
insgesamt:	255	47

Sechsmal wurden aus dem Stuhl von Kranken gleichzeitig *E. coli* und *Shigella* Stämme isoliert.

Die Eigenschaften der aus den Stuhlproben von 1014 Säuglingen und Kindern isolierten *E. coli*, *Klebsiella*, *P. mirabilis*, *P. vulgaris*, *P. morganii*, *Ps. aeruginosa* bzw. *Staphylococcus aureus* Stämme wurden untersucht. Von den Kranken litten 376 an enteralen, 638 an extraenteralen Erkrankungen.

In die erste Gruppe reichten wir diejenigen Kranken ein, in deren Stuhlproben bloß eine der vorher erwähnten Bakteriengruppen (*E. coli*, *Klebsiella*

usw.) gefunden wurden (272 Fälle). Die zweite Gruppe bildeten diejenigen enteralen Kranken, in deren Stuhlproben gleichzeitig mit den bekannten enteropathogenen Keimen auch die Stämme der von uns untersuchten Bakteriengruppen isoliert wurden (104 Fälle).

In vorliegender Arbeit werden unsere auf die *E. coli* Gruppe bezüglichen Beobachtungen beschrieben.

Material und Methoden

Während der einjährigen Beobachtungszeit isolierten wir von den Krankenhauspatienten aus der ersten bzw. wiederholten Fäkalkultur bei 602 Kranken insgesamt 967 *E. coli* Stämme. Aus einer Stuhlprobe wurden nach Möglichkeit 2–4 kolonimorphologisch verschiedene *E. coli* Stämme untersucht.

Isolierung der Stämme: Die Stuhlproben wurden bei jeder Gelegenheit auf Blutagar-Eosin-methylenblau-Desoxycholatcitrat- und Bismuthsulfitplatten geimpft. Zur orientierenden Identifizierung der gezüchteten Bakterien wurde der NOGRÁDY—RODLERSche [1] Polytrop-Nährboden benutzt, welcher vor allem die grundlegenden biochemischen Eigentümlichkeiten gut anzeigt.

Biochemische Untersuchungen: Zur Untersuchung der Fermentaktivität der Stämme verwendeten wir die von KAUFFMANN [2] empfohlenen Kohlenhydrate bzw. Alkohole (Adonit, Dulcit, Sorbit, Arabinose, Xylose, Rhamnose, Maltose, Salicin, Inosit, Lactose, Saccharose, Mannit, Glucose). Beobachtungsdauer bei 37°C 14 Tage. Die Indol-Methylrot und Voges-Proskauer-Proben wurden mit 3–5tägigen Kulturen angestellt. Die Urease-Probe auf Simmonschem Citrat-Nährboden wurde nach 4 Tagen abgelesen. Der Eijkman-Test wurde nach 48 Stunden (46°) abgelesen, die Gelatine-Verflüssigung und die Schwefelwasserstoffbildung bei 20°C nach 30 Tagen.

Serologische Untersuchungen: Die bei 100° 2 1/2 Stunden gekochten bzw. 1 Stunde autoklavierten Suspensionen der im Laufe der biochemischen Untersuchungen für *E. coli* qualifizierten Stämme wurden in einer Röhrenreihe mit einem coli »O« Gruppenserum agglutiniert, das mit 1–25 Standardstämmen von *E. coli* hergestellt worden war und zwar zunächst in polyvalenter Serummischung (von 1 bis 8), dann in entsprechenden monovalenten Seren, angefangen bei einer Röhrenverdünnung von 1 : 100 bis zum Titer der Seren. Die polyvalenten Serummischungen wurden aus Serumkomponenten gleichen Titors ausgewählt und in einer solchen Verdünnung angewendet, daß die störenden Kreuzagglutinationen nicht zur Geltung kommen. Der Vorgang wurde in einer früheren Arbeit beschrieben [3]. Wir betrachteten denjenigen *E. coli* Stamm als zu einer bestimmten »O« Gruppe gehörend, der bis zum Titer des gegebenen Serums agglutinierte.

Bestimmung der Empfindlichkeit gegen Antibiotika: Die Resistenz der isolierten *E. coli* Stämme gegen Antibiotika wurde mittels »Biotest« (Human, Budapest) Scheiben in der üblichen Weise auf Agarplatten bestimmt. Die auf die Scheiben aufgetragenen Antibiotika sind: Penicillin 30 IE, Streptomycin 30 µg, Oxytetracyclin 30 µg, Neomycin 100 µg, Polymyxin B 15 µg, Erythromycin 10 µg, Sulfonamide 400 µg. Die Ergebnisse wurden nach 20stündiger Inkubation bei 37°C abgelesen.

Ergebnisse

Fermentationsuntersuchungen: Die Fermentationsuntersuchung der 967 isolierten *E. coli* Stämme ergab bei der überwiegenden Mehrzahl der Stämme eine für die *E. coli* Gruppe charakteristische Vergärungsprobe. Zur Charakterisierung haben wir die Fermentationseigenheiten unserer in die 1–25 »O« Gruppen des diagnostischen Coli Antigenschemas eingereihten 197 Stämme zusammengefaßt (Tabelle II).

Wie aus Tabelle II ersichtlich, ist die Fermentationstätigkeit der *E. coli* Gruppe, ihre biochemische Aktivität bei weitem nicht einheitlich.

Die Abweichung von den charakteristischen Eigenschaften äußerte sich bei 18,8% der Stämme im Mangel an Indolbildung, bei 4,6% in der Citratver-

Tabelle II

Fermentationseigenschaften der untersuchten 197 in Serogruppen eingereihten E. coli Stämme

	Positiv		Negativ	
	Zahl	%	Zahl	%
Indol	160	81,2	37	18,8
Methylrot	197	100,0	—	—
Voges-Proskauer	—	—	197	100,0
Citrat	9	4,6	188	95,4
Urease	—	—	197	100,0
Gelatine	—	—	197	100,0
H ₂ S	—	—	197	100,0
Eijkman 46° C	150	76,1	47	23,9

wertung, bei 23,9% in der negativen Eijkmann-Reaktion. 197 Stämme haben sich in bezug auf Dulcit-, Sorbit-, Arabinose-, Xylose-, Maltose-, Lactose-, Mannit- und Dextroservergärung als einheitlich erwiesen. Rhamnose wurde durch 88,3%, Salicin durch 85,2%, Saccharose durch 30,9% der Stämme fermentiert. Adonit wurde von keinem der Stämme utlisiert. Die auf die Fermentaktivität der *E. coli* Stämme charakteristische Inositenbenützung konnte bei 3 Stämmen (1,5%) beobachtet werden. Der Mangel an Indolbildung, die Vermehrung auf Citrat, der negative Eijkmann-Test, ferner der Mangel der Rhamnose-, Salicin-, und Saccharosevergärung charakterisierten eine gewisse Gruppe der Stämme, namentlich den überwiegenden Teil der im fäkalen Material dominierenden Serogruppen (O : 2, O : 4, O : 8, O : 19).

Serologische Untersuchungen: Die auf Grund der Fermentationsuntersuchungen biochemisch als *E. coli* bestimmten 967 Stämme wurden einer serologischen Gruppenanalyse unterzogen. 395 Stämme stammten aus den Stuhlproben von 253 an Enterocolitis, 572 Stämme aus denjenigen von 349 an einer extraenteralen Erkrankung (Grippe, Bronchopneumonie, Otitis usw.) leidenden Säuglingen bzw. Kleinkindern über einem Jahr. In unserem Untersuchungsmaterial bildeten die extraenteralen Kranken die Kontrollgruppe.

Von den aus dem Stuhl der enteralen Kranken gezüchteten 395 *E. coli* Stämmen waren 93 von 81 Kranken (23,5%) in die 1—25 »O« Gruppe des diagnostischen *E. coli* Antigenschemas einzureihen. Von den aus den Stuhlproben der als Kontrolle verwendeten extraenteralen Kranken isolierten 572 *E. coli* Stämmen konnten 104, aus 93 Fällen stammenden Stämme (18,2%) in die 1—25 »O« Antigengruppe eingeordnet werden. Etwa 60% der serologisch identifizierbaren Stämme gehörten in 4 bzw. 3 unterschiedlichen Antigengruppen: in der Gruppe der enteralen Kranken ist das relativ häufige Vorkommen der Antigene O : 2, O : 4, O : 8 zu beobachten (Tabelle III).

Tabelle III

*Serologische Gruppenverteilung der isolierten 967 E. coli Stämme
(mit 1—25 E. coli Gruppenserien)*

Serogruppe »O«	Enterale Fälle (253 Kranke) (Stuhl) S t ä m m e		Extraenterale Fälle (349 Kranke) (Stuhl) S t ä m m e		Stämme insgesamt
	Zahl	%	Zahl	%	
1	4	4,3	4	3,9	8
2	10	10,7	16	15,3	26
3	3	3,2	3	2,9	6
4	22	23,6	34	32,7	56
5	2	2,2	4	3,9	6
6	2	2,2	4	3,9	6
7	6	6,5	5	4,8	11
8	13	14,0	15	14,4	28
9	—	—	—	—	—
10	3	3,2	2	1,9	5
11	6	6,5	4	3,9	10
12	—	—	—	—	—
13	—	—	2	1,9	2
14	—	—	—	—	—
15	6	6,5	1	0,9	7
16	—	—	—	—	—
17	3	3,2	3	2,9	6
18	—	—	—	—	—
19	10	10,7	5	4,8	15
20	—	—	—	—	—
21	2	2,2	—	—	2
22	—	—	—	—	—
23	1	1,0	2	1,9	3
24	—	—	—	—	—
25	—	—	—	—	—
In Serogruppen einge- reichte Stämme ins- gesamt	93	23,5	104	18,2	197
Nicht definierbare Stämme insgesamt ...	302	76,5	468	81,8	770
Insgesamt	395	100,0	572	100,0	967

Auf die kausale Rolle, welche die O : 2 und besonders die O : 4 Stämme spielen, auf ihre Dominanz in der Stuhlflora gesunder und an Enteritis leidender Säuglinge hat UJVÁRY [3] bereits hingewiesen. Bei unseren gegenwärtigen, vom Schauplatz der vorherigen Untersuchungen geographisch fernliegenden Spitalskrankengut gelangten wir zu ähnlichen Ergebnissen. In unseren vorliegenden Untersuchungen waren die aus den Stuhlproben der Kontrollgruppe stammenden O : 2 und O : 4 Stämme — im Verhältnis zur Gruppe der enteralen Kranken — in verhältnismäßig größerem Prozentsatz nachweisbar. Nach den Angaben der Tabelle III ließen sich die O : 2 Stämme in beiden Gruppen in gleichem Prozentsatz, die über Antigene O : 15 und O : 19 verfügenden Stämme aus dem Stuhl der an Enterocolitis leidenden Kranken in einem signifikant größerem Prozentsatz züchten. Von den 1—25 »O« Gruppenantigenen waren in der Gruppe der enteralen Kranken die »O« Antigene 9, 12, 13, 14, 16, 18, 20, 22, 24 und 25, in der Gruppe der extraenteralen Kranken (Kontrollen) die »O« Antigene 9, 12, 16, 18, 20, 21, 22, 24 und 25 nicht auffindbar.

Den Gegenstand unserer datenmäßigen Aufarbeitung bildeten jene an Enterocolitis leidenden 81 bzw. enteral symptomfreien 93 Kranken, aus deren Stuhl wir solche *E. coli* Stämme isolierten, die in die 1—25 »O« Gruppen des diagnostischen Antischemas eingereiht werden konnten. In Tabelle IV sind die Angaben der an Enterocolitis leidenden Kranken zusammengestellt.

Aus Tabelle IV geht hervor, daß die erste bzw. wiederholte Stuhluntersuchung von 52 Kranken pro Fall bloß die Züchtung eines in einer »O« Antigen-Gruppe gehörenden Stammes ergab. In den Stuhlproben von 17 Kranken gelang es, gleichzeitig mit einem der vorkommenden Serogruppen bekannte enteropathogene Keime (*Shigella*, *E. coli*), im Stuhl von 12 Kranken das Vorhandensein von 2 verschiedenen — in die 1—25 »O« Gruppen gehörenden — »O« Antigenen nachzuweisen. Die identifizierbaren *E. coli* Gruppenantigene kamen gleichzeitig mit bekannten enteropathogenen Keimen in folgender Verteilung vor; O : 1 = je 1 *Sh. flexneri* bzw. *Sh. sonnei*; O : 2 = 3 *Sh. sonnei*; O : 4 = 1 *Sh. sonnei*; O : 6 = 1 *E. coli* 111 : 4; O : 7 = 1 *Sh. sonnei*; O : 8 = 2 *Sh. sonnei*, je 1 *Sh. flexneri* bzw. *E. coli* 111 : 4; O : 11 = je 1 *Sh. flexneri* bzw. *Sh. sonnei*; O : 19 = 3 *Sh. sonnei*. Die *Shigella* wurde — abgesehen von einem Fall — ausschließlich aus dem Stuhl von über einem Jahr alten Kleinkindern isoliert. Dasjenige von den 2 verschiedenen aus einer Stuhlgruppe isolierten »O« Gruppenantigenen, das bei der Stuhluntersuchung auf Eosinmethylenblau-Plattennährboden in relativ geringerer Kolonienzahl anzüchtbar war, wurde in der entsprechenden Säule der Tabelle IV in Klammern gesetzt.

Klinische Beobachtungen: Aus dem Darmtrakt der Kranken waren in Reinkultur oder mit sonstigen »O« Gruppen gemischt, meist die O : 4 Coli Stämme nachweisbar (16 bzw. 5 Fälle). Die Mehrzahl der die O : 4 Stämme ausscheidenden Kranken bildeten 1—6 Monate alten Säuglinge die an leichter Gastro-Enterocolitis litten. Auf Grund der anlässlich der Aufnahme durchge-

Tabelle IV

Angaben der 81 an Enterocolitis leidenden Kranken

Serogruppe »O«	Zahl der Kranken, aus deren Stuhl			Zahl der Kranken insgesamt	Verteilung der Infektionen		Gastroenterocolitis			Alter der Kranken				
	ein »O« Antigen des <i>E. coli</i>	<i>E. coli</i> — be- kannter Entero- pathogen	auch eine sonstige »O« Gruppe des <i>E. coli</i>		Außen- welt	Kran- ken- haus	schwer	mittel- schwer	leicht	Trimenon				
										I.	II.	III.	IV.	über ein Jahr
wuchs														
1.	2	2	—	4	3	1	—	—	4	1	—	1	1	1
2.	5	3	1 (10)	9	9	—	—	2	7	—	—	—	—	9
3.	1	—	2 (5, 23)	3	2	1	—	1	2	—	1	—	1	1
4.	16	1	5 (7, 8, 11, 15, 15)	22	14	8	—	4	18	10	6	—	1	5
5.	1	—	—	1	1	—	—	—	1	—	—	—	—	1
6.	1	1	—	2	2	—	—	2	—	—	—	1	—	1
7.	3	1	—	4	4	—	—	1	3	—	—	—	—	4
8.	7	4	1 (2)	12	9	3	—	3	9	3	1	1	3	4
10.	1	—	—	1	1	—	—	1	—	1	—	—	—	—
11.	3	2	—	5	5	—	—	1	4	—	—	—	1	4
15.	3	—	—	3	2	1	—	1	2	2	1	—	—	—
17.	2	—	1 (15)	3	3	—	—	—	3	1	1	—	—	1
19.	6	3	1 (7)	10	8	2	—	3	7	2	1	1	1	5
21.	1	—	1	2	2	—	—	2	—	—	—	1	—	1
insgesamt	52	17	12	81	65	16	—	21	60	20	11	5	8	37

Die Zahlen in Klammern bezeichnen die »O« Antigene der als zweite isolierten Stämme

fürten bakteriologischen Stuhluntersuchung konnte festgestellt werden, daß $\frac{4}{5}$ der Kranken ihren *E. coli* Stamm außerhalb des Krankenhauses aquirierten. Bloß bei $\frac{1}{5}$ der Kranken ließ sich eine Spitalsinfektion nachweisen, die durch das Erscheinen der *E. coli* Stämme im Stuhl und durch die gleichzeitigen oder nachfolgenden enteralen Symptome begleitet war. In 50% der aus den Hausdyspepsien stammenden Stuhlproben (8 Fälle) war ebenfalls die O : 4 Sero-Gruppe auffindbar. Die Hausinfektionen zeigten aber kein gehäuftes Vorkommen. 75% der außerhalb und innerhalb des Krankenhauses erworbenen Dyspepsien liefen in Form einer leichten sich auf 5–12 Behandlungstage erstreckenden Gastro-Enterocolitis bzw. Enterocolitis ab. Mittelschwere enterale Symptome (Fieber bis 39°C, mehrmaliges Erbrechen, täglich 6–8 flüssige, wäßrige bzw. schleimige Stuhlentleerungen, Gewichtsabnahme usw.) traten bei 21 Kranken auf. Als ätiologischer Faktor stand im Hintergrund der Symptome in der Mehrzahl der Fälle primär eine Shigella oder *E. coli* 111 : 4 Infektion. Die Schwere der Krankheitsform oder die Ausbildung eines toxischen Symptomenkomplexes haben wir nicht beobachtet. Mit gezielter antibiotischen Therapie, wobei in erster Linie die perorale bzw. i.m. Dosierung von Chloramphenicol und Oxytetracyclin angewendet wurde, kombiniert mit Allgemeinbehandlung (Ringer-Dextrose, Plasma, Vitamine), erzielten wir rasche klinische Heilung.

Das Auftreten der Symptome der Enterocolitis war in 2 Altersgruppen, bei 31 Säuglingen im I–II. Trimenon und bei 37 1–4 Jahre alten Kleinkindern am häufigsten. Aus Tabelle IV geht auch hervor, daß die Stämme der Sero-Gruppe O : 4 zum überwiegenden Teil aus dem Stuhl der 0–6 Monate alten Kranken und die Stämme O : 2 ausschließlich bei 1–4 Jahre alten Kleinkindern isoliert wurden. Sonstige »O« Antigene verteilten sich je nach Krankheitsgruppen.

Bei nosokomialen Kreuzinfektionen isolierten ØRSKOV, ØRSKOV und PAERREGAARD [4] aus dem pathologischen und normalen Stuhl von 43 Kindern eines dänischen Provinz-Kinderheimes am häufigsten die Sero-Gruppen O : 118, O : 21 und O : 68. Im Laufe ihrer 3 Monate lang dauernden Beobachtungen im Krankensaal A und B des Kopenhagener Kinderspitals konnten sie das Vorkommen von 23 bzw. 31 unterschiedlichen *E. coli* »O« Antigenen und die Kreuzinfektion der Kinder mit einigen der Stämme wahrnehmen.

Für die saisonale Gestaltung der außerhalb des Krankenhauses erworbenen und der nosokomialen Enterocolitiden ist eine frühsummerliche (Mai–Juni–Juli) und eine frühherbstliche (September–Oktober) Kulmination charakteristisch. Von unseren 81 enteralen Kranken erkrankten 28 in der Sommerperiode, 45 in der Herbstperiode.

Die Stuhlflora der enteral symptomfreien 93 Säuglingen bzw. Kleinkindern wurde in etwa 30% der Fälle teils in Reinkultur (31 Fälle), teils in Mischkultur von Gruppe O : 4 Stämme gebildet (Tabelle V).

Tabelle V
Angaben von 93 Kontrollkranken

Serogruppe: »O«	Zahl der Kontrollen aus deren Stuhl			Zahl der Kontrollen insgesamt	Alter				
	ein »O« Antigen des <i>E. coli</i>	<i>E. coli</i> bekannter Entero- pathogen	auch eine sonstige »O« Gruppe des <i>E. coli</i>		T r i m e n o n				über ein Jahr
					w u c h s	I.	II.	III.	
1.	2	—	—	2	—	—	1	—	1
2.	11	—	3 (5, 5, 5)	14	—	6	3	1	4
3.	3	—	—	3	—	1	1	1	—
4.	31	—	3 (2, 8, 19)	34	2	12	9	4	7
5.	1	—	—	1	—	—	1	—	—
6.	4	—	—	4	—	1	—	1	2
7.	5	—	—	5	—	—	1	1	3
8.	11	—	3 (1, 1, 2)	14	1	1	3	—	9
10.	2	—	—	2	—	—	—	1	1
11.	4	—	—	4	2	1	—	—	1
13.	1	—	—	1	—	—	—	1	—
15.	1	—	—	1	—	—	—	—	1
17.	2	—	—	2	1	—	1	—	—
19.	3	—	1 (13)	4	—	—	—	1	3
23.	1	—	1 (17)	2	1	—	—	—	1
insgesamt	82	—	11	93	7	22	20	11	33

Die Zahlen in Klammern bezeichnen die »O« Antigene der als zweite isolierten Stämme

Neben der Dominanz der O : 4 Stämme war in der Kontrollgruppe — ähnlich wie in der Enterocolitisgruppe — die Häufigkeit der O : 2 und O : 8 Stämme (aus je 14 Fällen) augenfällig. Antigen O : 2 wurde zusammen mit Antigen O : 5 aus dem Stuhl von 3 Säuglingen isoliert. Das gleichzeitige Vorkommen des O : 8 Stammes mit Stamm O : 1 konnten wir in 2 Fällen beobachten. Neben den im Stuhl dominierenden »O« Gruppenantigenen kamen die Stämme 1—25 »O« ziemlich selten vor.

Das Alter der Kontroll-Säuglinge zeigte gegenüber der Verteilung der an Enterocolitis leidenden Säuglinge eine entschiedene Verschiebung in die Richtung der höheren Lebensmonate (II., III., IV. Trimenon). Während in der Gruppe der an Enteritis erkrankten Säuglinge diejenigen, die in das I. Trimenon gehören 25% der untersuchten Fälle ausmachten (20 Fälle), war die Zahl der Säuglinge gleichen Alters in der Kontrollgruppe die geringste (7 Fälle 7,5%). Auch die »O« Antigengruppe der aus den Stuhlproben der Enterocolitisgruppe und der Kontrollgruppe der 0—3 Monate alten Säuglinge isolierten *E. coli* Stämme war

verschieden. Eine Ausnahme bildeten die Serogruppen O : 4 und O : 8, doch ließen sich diese bei wesentlich mehr an Dyspepsie leidenden Säuglingen (10 bzw. 3 Fälle) als bei den Kontrollen (2 Fälle bzw. 1 Fall) züchten.

Infolge technischer Schwierigkeiten konnten wir keine systematischen Untersuchungen in der Richtung durchführen, die Stuhlproben unserer Kranken — vom Gesichtspunkt des Vorkommens der *E. coli* Serogruppen — vor der Ausbildung der Symptome der Enterocolitis, während deren Verlauf oder nach Abklingen der Symptome zu analysieren. Unsere Beobachtungen beschränkten sich auf die systematische Untersuchung der beim Auftreten der Symptome der Enterocolitis und nach den darauffolgenden einigen Tagen gewonnenen Stuhlproben. Trotzdem gelang es uns in einigen Fällen, die Gestaltung der Darmflora während des ganzen Verlaufs der Enterocolitis zu beobachten.

B.Z. 3 Monate alter Säugling wurde mit Gastroenterocolitis-Symptomen aufgenommen. Der Säugling war zu Hause auf gemischte Diät (Muttermilch + Kuhmilchernährung) eingestellt. Die am Tage nach der Aufnahme erfolgte Stuhluntersuchung ergab *E. coli* O : 4 und O : 15. Nach 5tägiger Chloramphenicoltherapie wurde der Säugling symptomfrei und nach dem dritten Tag der Behandlung konnte Stamm O : 15 nicht mehr isoliert werden. Bei der Entlassung am 8. Tag enthielt der Stuhl neben *E. coli* O : 4 vorwiegend Klebsiella. Es ist bemerkenswert, daß bei Anwendung von Breitspektrumantibiotika meistens die Klebsiella dominierte.

Aus den Untersuchungen von SEARS und BROWNLEE [5] bzw. SEARS, BROWNLEE und UCHIYAMA [6] ist bekannt, daß die Darmflora der Säuglinge aus *E. coli* Stämmen sog. »ständigen« und »vorübergehenden« Charakters gebildet wird. Die Verfasser klassifizierten solche Serotypen von *E. coli* als »ständige« Stämme, die im Dickdarm längere Zeit (z. B. 1 Jahr lang), als »vorübergehende« hingegen solche, die im Stuhl nur kurze Zeit hindurch nachweisbar waren. In unserem Material waren als »ständige« Stämme die aus dem normalen Stuhl in bedeutender Zahl züchtbaren *E. coli* Stämme O : 2, O : 4 und O : 8 zu betrachten. Die »vorübergehenden« Stämme gelangen in erster Linie mit der Nahrung in den Magen-Darmtrakt und waren im Stuhl nur kurze Zeit nachweisbar. Falls »ständige« Stämme in den Darmtrakt von Säuglingen gelangen, in denen sie nicht heimisch sind, d.h. nicht der normalen Darmflora angehören — wie z. B. bei Krankenhaus-Kreutzinfektionen —, übernehmen sie die Rolle der »vorübergehenden« Stämme. Nach unserer Annahme können die in den Magen-Darmtrakt gelangten »vorübergehenden« *E. coli* Stämme infolge Änderung der Lebensverhältnisse des Säuglings (Änderung der Zusammensetzung der Nahrung, die in Verbindung mit den Krankheiten eingetretene Abnahme der Resistenz des Organismus, Milieu usw.) gegebenenfalls zu kausalen Faktoren der Magen-Darmkatarrhen des Säuglingsalters werden.

Untersuchung der Antibiotikumempfindlichkeit: Die isolierten *E. coli* Stämme wiesen eine hochgradige Antibiotikumempfindlichkeit auf. Wir zogen die Gruppe der empfindlichen und die der mäßig empfindlichen zusammen. Wir gruppierten gesondert die in die 1—25 »O« Gruppe einreihbaren sowie die nicht einteilbaren Stämme. Die Ergebnisse der Empfindlichkeitsuntersuchungen sind in Tabelle VI sichtbar.

Tabelle VI

Antibiotikumempfindlichkeit der isolierten 967 E. Coli Stämme

Antibiotikum	In die 1—25 »O« Gruppen eingereihte Stämme				In die 1—25 »O« Gruppen nicht einreihbare Stämme			
	empfindlich		resistent		empfindlich		resistent	
	Zahl	%	Zahl	%	Zahl	%	Zahl	%
Penicillin	—	—	197	100,0	—	—	770	100,0
Streptomycin	180	91,4	17	8,6	692	89,9	78	10,1
Chloramphenicol	151	76,6	46	23,4	620	80,5	150	19,5
Chlortetracyclin	98	49,7	99	50,3	577	74,9	193	25,1
Oxytetracyclin	139	70,5	58	29,5	631	81,9	139	18,1
Neomycin	197	100,0	—	—	770	100,0	—	—
Polymyxin B	197	100,0	—	—	763	99,1	7	0,9
Erythromycin	195	99,0	2	1,0	506	65,7	264	34,3
Sulfonamid	—	—	197	100,0	50	6,5	720	93,5

Ein gegen Penicillin empfindlicher Stamm wurde erwartungsgemäß nicht gefunden. Auffallend war der hohe Prozentanteil der streptomycinempfindlichen Stämme (91,4% bzw. 89,9%), was vermutlich mit der heute schon seltenen Anwendung des Streptomycins bei Darminfektionen zusammenhängt. Chloramphenicol und Oxytetracyclin erwiesen sich im Falle der in Serogruppen einreihbaren und der nicht definierbaren Stämme als fast identisch und zugleich von gutem Effekt (76,4%, 70,5% bzw. 80,5%, 81,9%). Hochgradige Toleranz gegen Chlortetracyclin war besonders für die in Serogruppen einreihbaren Stämme charakteristisch (50,3%). Dagegen kam kein gegen Neomycin resistenter Stamm vor. Polymyxin B und Erythromycin übten in vitro auf einen großen Prozentsatz eine Hemmwirkung aus (100%, 99,1% bzw. 99%, 65,7%), doch äußerte sich dieser Effekt meist in der mäßigen Empfindlichkeit der Stämme. Gegen Sulfonamide waren 100% der in die 1—25 Serogruppen gehörenden *E. coli* und 93,5% der nicht definierbaren Stämme resistent. 6,5% der Stämme waren gegenüber Sulfonamide mäßig empfindlich.

Von den serologisch identifizierbaren »O« Stämmen griffen wir die dominierenden Stammtypen heraus, um Aufschluß über die Antibiotikumemp-

findlichkeit und den etwaigen Zusammenhang zwischen Empfindlichkeit und Antigenstruktur zu erhalten. Die Empfindlichkeit der aus Enterocolitisfällen isolierten O : 2, O : 4, O : 8 und O : 9 Stämme zeigte keine wesentliche Abweichung von derjenigen der aus der Kontrollgruppe stammenden identischen »O« Gruppen, weshalb wir diese gemeinsam behandeln. Das Ergebnis der Antibiotikumempfindlichkeits-Untersuchungen enthält Tabelle VII.

Tabelle VII

Antibiotikumempfindlichkeit der von enteralen Kranken und Kontrollen isolierten prädominierenden E. coli Serogruppen

Antibiotikum	Gruppe O : 2 (26 Stämme) %		Gruppe O : 4 (56 Stämme) %		Gruppe O : 8 (28 Stämme) %		Gruppe O : 19 (15 Stämme) %	
	E	R	E	R	E	R	E	R
Penicillin	—	100,0	—	100,0	—	100,0	—	100,0
Streptomycin ..	100,0	—	80,4	19,6	96,4	3,6	100,0	—
Chloramphenicol	100,0	—	26,8	73,2	100,0	—	93,3	6,7
Chlortetracyclin	80,7	19,3	35,7	64,3	53,6	46,4	60,0	40,0
Oxytetracyclin .	80,7	19,3	26,8	73,2	96,4	3,6	93,3	6,7
Neomycin	100,0	—	100,0	—	100,0	—	100,0	—
Polymyxin B ..	100,0	—	100,0	—	100,0	—	100,0	—
Erythromycin ..	100,0	—	98,2	1,8	100,0	—	100,0	—
Sulfonamid	—	100,0	—	100,0	—	100,0	—	100,0

Zeichenerklärung:

E = empfindlich + mäßig empfindlich

R = resistent

Die O : 2 Gruppenstämme zeichneten sich mit ihrer Polysensitivität aus. Neben ihrer 100%igen Resistenz gegen Penicillin und Sulfonamide — welche Eigenschaft sich auch mit derjenigen der Stämme anderer Serogruppen konsequent paarte — erwies sich bloß eine kleine Gruppe der O : 2 Stämme (19,3%) chlor- und oxytetracyclinresistent. Eine ähnliche Empfindlichkeit war auch bei den Serogruppen O : 8 und O : 19 zu beobachten, bloß war der prozentuale Anteil der gegen Chlortetracyclin resistenten und gegenüber Oxytetracyclin empfindlichen Stämme höher. Die überwiegende Mehrzahl der Stämme O : 4 war durch ihre Toleranz gegen Chloramphenicol, Chlor- und Oxytetracyclin charakterisiert (73,2%, 64,3%, 73,2%). 95% der in die 1—25 Gruppe eingereihten gegen Chloramphenicol resistenten Stämme, 34% der gegen Chlortetracyclin resistenten und 80% der gegen Oxytetracyclin resistenten Stämme gehörten zu der O : 4 Gruppe. Die gegen Streptomycin resistenten Stämme waren sozusagen bloß in dieser Serogruppe zu finden (19,6%) und

bildeten 64,7% der serologischen, identifizierbaren, gegen Streptomycin resistenten Stämme.

Unsere Beobachtungen in Verbindung mit der O : 4 Gruppe werden durch die Angaben unserer früheren Untersuchungen unterstützt, wonach sich von den aus den Infektionen der Harnwege isolierten *E. coli* O : 4 Stämmen 54,8% gegen Streptomycin und je 50% gegen Chloramphenicol, Chlor- und Oxytetracyclin als resistent erwiesen [7]. Heute ist dieser prozentuale Anteil noch höher, was mit der allgemeinen Anwendung der Breitspektrumantibiotika — in erster Linie von Chloramphenicol und Oxytetracyclin — in Zusammenhang gebracht werden kann. Neomycin übte — ohne Rücksicht auf die Serogruppe — eine ausdrückliche Hemmwirkung aus; auch klinisch übertraf es den Effekt sonstiger Antibiotika.

Die in die 1—25 »O« Gruppe nicht einreihbaren 770 *E. coli* Stämme konnten auf Grund ihrer Empfindlichkeit bzw. Resistenz gegen Antibiotika in 23 Antibiogrammtypen gruppiert werden. 55,6% bzw. 64,3% der von enteralen Kranken und Kontrollen isolierten Stämme gehörten gleicherweise 3 unterschiedlichen Antibiogrammtypen (Typ 1, 2 und 11) an. Für die Stämme des 1. Antibiogrammtyps war Resistenz gegen Penicillin und Sulfonamide, für die des zweiten Resistenz gegen Penicillin, Sulfonamide und Chlortetracyclin, für die des 11. Antibiogrammtyps Resistenz gegen Penicillin, Sulfonamide und Erythromycin charakteristisch. Die Verteilung der Antibiogrammtypen der nicht definierbaren *E. coli* Stämme ist in Tabelle VIII wiedergegeben.

Tabelle VIII

Verteilung der Antibiogrammtypen der mit 1—25 *E. coli* »O« Serum nicht definierbaren *E. coli* Stämme in der Kranken- und Kontrollgruppe

Antibiogrammtypen	Enterale Kranken (302 Stämme) %	Extraenterale Kranken (468 Stämme) %
1	31,5	33,3
2	7,9	7,9
11	16,2	23,1
Typ 1, 2, 11 insgesamt:	55,6	64,3
sonstige	44,4	35,7
insgesamt:	100,0	100,0

Die mehr als 50% der Stämme ausmachenden Antibiogrammtypen 1, 2 und 11 waren bei Kranken und Kontrollen pro Typ in gleicher bzw. nahezu

gleicher Verteilung zu finden. In beiden Gruppen kommen besonders die polysensitiven — zum 1. Antibiotigrammtyp gehörenden — Stämme am häufigsten vor (31,5% bzw. 33,3%). Die Mehrzahl der O : 2, O : 4, O : 8 und O : 19 Stämme war durch eine konsequente Empfindlichkeit charakterisiert, was auf einen Zusammenhang zwischen Antigenstruktur und Antibiotikumempfindlichkeit hinweist. Wie bei den 1–25 *E. coli* »O« Gruppen, scheinen auch hier etwa 65% der serologisch nicht definierbaren *E. coli* Stämme auf Grund des charakteristischen Antibiotigrammtyps bloß zu einigen der »O« Gruppenantigene höherer Numerierung gehören.

Besprechung

Auf dem Gebiete der Aufklärung der ätiologischen Faktoren der infektiösen Magen- und Darmkatarrhe des Säuglings- und Kleinkindesalters, ebenso wie im Bereich ihrer präventiven und therapeutischen Abwehr kamen wir im letzten Jahrzehnt mit einem bedeutenden Schritt vorwärts. Die günstige Gestaltung der Säuglingsmortalität kann z. T. als Widerspiegelung der mit den Darminfektionen im Zusammenhang stehenden Forschungsarbeit registriert werden. Das Erkennen der pathogenetischen Rolle der über »B« Antigene verfügenden *E. coli* Stämme vermehrte wohl die Zahl der bakteriologisch verifizierbaren Krankheitsfälle weiter, doch blieb die Identität des Krankheitserregers bei etwa 50% der Fälle der Säuglingsdyspepsien ungeklärt. Wir richteten daher unsere Aufmerksamkeit auf die Klärung der pathogenetischen Rolle der im Stuhl vorkommenden verschiedenen Darmbakteriengruppen.

Es wurde festgestellt, daß in der Dickdarmflora der Säuglinge und Kleinkinder zu einigen 1–25 Serogruppen gehörenden *E. coli* Stämme vorkommen. Die dominierenden »O« Gruppenantigene (O : 2, O : 4, O : 8) waren bei den enteralen Kranken und den Kontrollen — die Verteilung nach Lebensalter außer acht lassend — in annähernd gleicher prozentualer Verteilung zu finden. Eine Ausnahme bildeten die Gruppenantigene O : 19 und O : 15, welche sich aus dem Stuhl enteraler Kranken öfters isolieren ließen. Die im Stuhl dominierenden *E. coli* »O« Serogruppen sind sicherlich als sog. »ständige« Glieder der normalen Dickdarmflora des Säuglings und Kleinkindes zu betrachten. Die als »vorübergehend« bezeichnete — im Stuhl periodisch vorkommende und kürzere Zeit nachweisbare — *E. coli* Stämme gehören in mehrere »O« Antigengruppen, ihr Erscheinen im Stuhl ist gewöhnlich mit Milieuveränderung, der Umstellung von Nahrung und Diät, mit der in Krankenhaus-Kollektiven oft zustandekommender Kontaktinfektion zu erklären. Die Darminvasion mit vorübergehenden Stämmen kann auch durch irgendeine, die Widerstandsfähigkeit des Organismus herabsetzende Krankheit günstig beeinflusst werden. Es ist denkbar, daß auch die »ständigen« *E. coli* Stämme der normalen Dickdarmflora zu Stämmen »vorübergehenden« Charakters werden z. B. im Darmtrakt

solcher Säuglinge, die über den neu aquirierten »ständigen« Stamm bisher nicht verfügten. Unter gegebenen Umständen kann die Vermehrung des frisch erworbenen Stammes zur Verschiebung der Darmflora und infolgedessen zur Ausbildung enteraler Symptome führen. Besonders die labile, in Entwicklung begriffene Darmflora der Säuglinge im ersten Lebensmonat kann günstige Bedingungen zur Vermehrung eines neueren *E. coli* Stammes schaffen.

Diese Überlegung scheint auch unsere Beobachtung zu unterstützen, wonach von enteralen Infektionen in erster Linie die 1–3 Monate alten Säuglinge betroffen werden und daß diejenigen zum I. Trimenon gehörenden an Enteritis leidenden Säuglinge aus deren Stuhlproben die O : 4 Stämme »ständigen« Charakters isoliert wurden, in größerer Zahl vertreten sind, als die *E. coli* Stämme ausscheidenden Kontrollen gleichen Alters, oder z. B. die an Durchfall leidenden, mehrere Lebensmonate alten Säuglinge. Nach unseren Beobachtungen dürften die »vorübergehenden« *E. coli* Stämme in den Enterocolitiden des Säuglingsalters vermutlich eine kausale Rolle spielen.

Trotz der entschiedenen Saisonmäßigkeit (Frühjahr, Herbst) der im Laufe unserer Untersuchungen beobachteten enteralen Erkrankungen gelang es bloß in etwa 20% der Fälle gleichzeitig mit irgendeinem *E. coli* Stamm die Gegenwart eines bekannten enteropathogenen Keimes nachzuweisen. Von 80% des Stuhles der untersuchten Kranken erhielten wir eine *E. coli* Reinkultur, die meist zu einer oder zwei verschiedenen Serogruppen gehörte. Als prädisponierender Umstand kann angesehen werden, daß die enteralen Erkrankungen auf Grippeperioden fielen. Die saisonale Gestaltung der Enterocolitiden ist in sich allein auf Kreuzinfektionen nicht zurückzuführen, da $\frac{4}{5}$ der Darminfektionen ohne Zweifel außerhalb des Krankenhauses erworben wurden. Das sporadische Vorkommen der Mehrzahl der Enterocolitiden dient zwar nicht als idealer Stützpunkt in bezug auf die *E. coli*-Ätiologie der Darmsymptome, doch zeugt der Erfolg der adequadaten antibiotischen Therapie von der fakultativ enteropathogenen Rolle der Stämme der *E. coli* Gruppe.

Verglichen mit der Antibiotikumresistenz in vitro der aus früheren Jahren stammenden *E. coli* Stämme, zeichneten sich die gegenwärtigen Stämme mit ihrer prozentual höheren Empfindlichkeit gegen Streptomycin und ihrer Toleranz gegen Chloramphenicol aus. In bezug auf die Resistenz gegen die üblichen Antibiotika sind besonders die zur Serogruppe O : 4 gehörenden Stämme hervorzuheben. In der Empfindlichkeit der isolierten *E. coli* Stämme gegen Neomycin ist im Verlauf der vergangenen 10 Jahre — ohne Rücksicht auf die Serogruppe — keine Änderung eingetreten.

Laut unserer früheren und gegenwärtigen Untersuchungen scheint die in den Gastroenterocolitiden des Säuglingsalters gespielte fakultativ enteropathogene Rolle der Stämme der *E. coli* Gruppe — der *Coli dyspepsiae* Typen ähnlich — infolge der Beteiligung irgendeines prädisponierenden Faktors und der Änderung der normalen Coliflora des Dickdarmes wahrscheinlich zu sein.

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BEOBSACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

II. UNTERSUCHUNG DER ROLLE DER KLEBSIELLA-STÄMME

Von

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Zusammenfassung. 1. Bei einem Krankenmaterial von 1034 Fällen konnten wir 304 Klebsiella-Stämme isolieren. Es gelang, 37,4% der Stämme in einem der Typen 1–30 des Klebsiella Antigenschemas einzuordnen.

2. Am häufigsten kamen die Typen K : 28, K : 2 und K : 14 vor.

3. In Reinkultur wurde Klebsiella bei 10 schweren, 41 mittelschweren und 82 leichten Fällen von Enterocolitis isoliert.

4. Klebsiella vom Typ K : 28 verursachte in 2 Fällen eine kleinere Hausepidemie.

5. Auf Grund der Angaben kann die fakultativ enteropathogene Rolle der Klebsiella-Gruppe in den Gastroenterocolitiden des Säuglings- und Kindesalters als wahrscheinlich betrachtet werden.

Die pathogenetische Rolle der Klebsiellen wurde in erster Linie in Verbindung mit Erkrankungen der Luftwege, der Harnwege und sonstiger extraenteraler Infektionen bekannt. Im Darmkanal, ferner in Verbindung mit Gastroenteritiden wurde ihre Rolle sozusagen kaum untersucht. Als Konklusion unserer früheren Untersuchungen [1] tauchte der Gedanke auf, die Erreger der Dyspepsien des Säuglings- und Kindesalters unbekannter Herkunft unter den im Darmtrakt auch unter normalen Verhältnissen auffindbaren Darmbakteriengruppen und so auch unter den Stämmen der Klebsiella-Gruppe zu suchen. Zur Prüfung der Frage ist die serologische Analyse die Methode der Wahl, die die Prüfung der Rolle der Klebsiella-Typen an Kranken und gesunden Personen, ferner in verschiedenen Krankheitsbildern, namentlich auch in den Darminfektionen des Säuglings- und Kindesalters ermöglicht.

KAUFFMANN [2] sowie ØRSKOV [3] isolierten aus dem Urin von Cystitis-kranken Klebsiella-Stämme vom Antigenzeichen K : 8, 9, 10 und BROOKE [4] die Serotypen K : 7, 8, 9, 10. Bei Infektionen der Luftwege beobachteten EDWARDS und FIFE [5] das häufigere Vorkommen der Typen K : 1, 2, 4, ØRSKOV [6] der Typen K : 1, 3, 4, 16, 26, 29, 32, 41, 47 und 68. UJVÁRY [1] isolierte aus enteralem und extraenteralem Krankenmaterial den Klebsiella-Stamm K : 16 am häufigsten. SAKAZAKI und NAMIOKA [7] fanden im Sputum vorwiegend die Typen K : 1, 2, 3, 4, und im Urin Typ K : 10. Im Stuhl und Urin kamen fast alle Serotypen vor. ØRSKOV, ØRSKOV und PAERREGAARD [8]

isolierten anlässlich einer in zwei Krankensälen eines kopenhagener Kinderspitals aufgetretenen Hausepidemie von *E. coli* und Klebsiella Dyspepsie aus dem Stuhl der Kranken die Klebsiella-Serotypen K : 16, 18, 20, 26, 27, 34, 53, 54 und 55.

Material und Methoden

Die Aufarbeitung des untersuchten Materials und das Sammeln der Stämme wurde bereits beschrieben [11].

Die *biochemischen Teste* (Indol, Methylrot, Voges-Proskauer R., Citrat-Utilisation, Ureumspaltung, Dulcit-, Adonit-, Inosit-Fermentation) wurden nach der Vorschrift von KAUFFMANN [9] durchgeführt.

Die *serologischen Untersuchungen* wurden mit den durch die Klebsiella Standardstämme K : 1—K : 30 gewonnenen »OK« Kaninchenimmunsere angestellt. Die Typen wurden auf Grund der »K«-Antigene bestimmt. Die Rörchenagglutination wurde zunächst in polyvalenten Serum-mischungen (3 polyvalenten und 2 monovalenten Seren), dann in den die polyvalenten Seren bildenden Typenseren vorgenommen [1].

Die *Antibiotikumresistenz* wurde mit Diffusionspapierscheiben (HUMAN, Budapest) bestimmt.

Ergebnisse

Tabelle I zeigt die fermentativen Eigenschaften der aus dem Stuhl von 290 Säuglingen und Kleinkindern isolierten 304 Klebsiella-Stämmen.

Tabelle I

Reaktion	Fermentation der isolierten 304 Klebsiella-Stämme	
	Positiv	Negativ
Indol	10	294
Methylrot	16 3±	285
Voges-Proskauer	293	11
Citrat	302	2
Urease	304	—
Inosit	301	3
Adonit	290	14
Dulcit	50	254

Die überwiegende Mehrzahl der Stämme ließ sich in die für die Klebsiellen charakteristische übliche IMVC Fermentationsformal: — — + + einfügen. Eine Abweichung von der typischen Gärung haben wir im Falle von 10 indolbildenden, von 19 Methylrot-Reaktion produzierenden, von 11 Voges-Proskauer und von 2 Citrat negativen Stämmen beobachtet. Urease wurde von sämtlichen Stämmen gebildet.

Von den Kohlenhydraten untersuchten wir die Fermentation von Inosit, Adonit und Dulcit. Wir konnten bei 3 Stämmen das Fehlen der Inositgärung, bei 14 Stämmen das Fehlen der Adonitgärung beobachten. Dulcit wurde von 50 Stämmen gespalten.

Die identifizierten Klebsiellen wurden einer serologischen Typenanalyse unterworfen. Es gelang von 304 Stämmen, die aus 290 klinischen Fällen stammten, 112 (36,9%) in einen der Typen K : 1—30 einzuordnen, während 189 (62,2%) vermutlich in die durch uns nicht untersuchten Typen 31—72 gehörten. Zwei Stämme agglutinierten spontan, ein Stamm erwies sich als K minus-Variant. Die serologische Typenverteilung veranschaulicht Tabelle II.

Tabelle II

Serologische Typenverteilung der isolierten 304 Klebsiella-Stämme

Serotyp »K«	Enterale Kranken (Stuhl)		Extraenterale Kranken (Stuhl)		Insgesamt	
	Zahl	%	Zahl	%	Zahl	%
2	10	14,9	10	22,2	20	17,8
3	3	4,5	1	2,2	4	3,6
5	1	1,5	—	—	1	0,9
10	1	1,5	—	—	1	0,9
13	2	3,0	1	2,2	3	2,7
14	6	8,9	4	9,0	10	9,0
15	2	3,0	1	2,2	3	2,7
18	1	1,5	7	15,5	8	7,2
20	2	3,0	1	2,2	3	2,7
21	2	3,0	4	9,0	6	5,3
23	1	1,5	2	4,4	3	2,7
24	3	4,5	3	6,7	6	5,3
26	4	6,0	2	4,4	6	5,3
27	1	1,5	1	2,2	2	1,8
28	23	34,3	5	11,1	28	25,0
30	5	7,4	3	6,7	8	7,2
In Typen eingeordnet	67	37,4	45	36,0	112	36,9
Nicht definierbar	109	61,0	80	64,0	189	62,2
K minus-Variant und agglutinierte spontan	3	1,6	—	—	3	0,9

Wir beobachteten das Vorkommen von 16 verschiedenen K Typen. Von den Kranken isolierten wir 179 Stämme; von diesen konnten 67 (37,4%) in einen der Typen 1—30 eingereiht werden. Außer den nicht einreihbaren 109 Stämmen (61,0%) agglutinierten zwei spontan, einer war ein K minus-Variant

(1,6%). Aus den 125 Stämmen der Kontrollgruppe gehörten 45 Stämme (36%) zu den Typen 1–30; die übrigen 80 (64%) konnten nicht identifiziert werden. In der Gruppe der enteralen Kranken beobachteten wir das Vorkommen von 16, in der Kontrollgruppe das von 14 Typen. In der letzteren kamen nämlich die Typen K : 5 und K : 10 nicht vor. Aus dem Stuhl der Kranken und der Kontrollen waren die Serotypen mit Ausnahme des K : 28 in fast gleichem Prozentsatz nachweisbar. Dies wurde durch die statistische Berechnung auch bekräftigt, die Verteilung der aus den beiden Gruppen isolierten Typen erwies sich nämlich in hohem Grade homogen ($\chi^2_{15} = 150,3$ $P < 0,01$). Am häufigsten wurden die Serotypen K : 28 (25,0%), dann K : 2 (17,8%) und K : 14 (9,0%) isoliert. Beachtenswert war das Vorkommen des Typs K : 28, der im Krankematerial in signifikant größerer Zahl vorkam, als in den Kontrollstühlen ($\chi^2 = 4,86$ $0,05 < P < 0,02$).

Die Untersuchung der Antibiotikumempfindlichkeit zeigte eine hochgradige Resistenz gegen die üblichen Antibiotika. Penicillin-, sulfonamid- und bacitracinempfindliche Stämme kamen nicht vor. Sämtliche Stämme waren neomycin- und polymyxin-B-empfindlich. Gegenüber Streptomycin waren 43,7%, gegen Chloramphenicol 27,2%, gegen Chlortetracyclin 59,8%, gegen Oxytetracyclin 45,3% und gegen Erythromycin 10% empfindlich oder mäßig empfindlich. Mit den Beobachtungen der früheren Jahre verglichen erwies sich die Zahl der chloramphenicolresistenten Stämme auffallend hoch (72,8%), vermutlich infolge der langjährigen allgemeinen Anwendung dieses Antibiotikums.

Zwecks Analyse der erzielten Angaben haben wir auch das Antibio-
gramm einiger öfters vorkommenden Serotypen zusammengestellt (Tabelle III).

Tabelle III

*Empfindlichkeit der isolierten Klebsiella-Stämme gegenüber Streptomycin,
Chloramphenicol und Tetracyclin*

(Zahl der geprüften Stämme: 301)

Serotyp »K«	Zahl der Stämme	Streptomycin		Chloram- phenicol		Tetracyclin	
		E	R	E	R	E	R
2	20	14	6	2	18	6	14
14	10	4	6	2	8	5	5
28	28	4	24	3	25	26	2
Sonstige Typen	54	20	34	16	38	35	19
ND-Stämme	189	82	107	53	136	96	93

Zeichenerklärung: E = empfindlich
R = resistent

In Tabelle III wurden diejenigen Antibiotika nicht angeführt, gegen die sich die isolierten Stämme ausnahmslos empfindlich (Neomycin, Polymyxin B) bzw. resistent (Penicillin, Sulfonamiden, Bacitracin) erwiesen. Streptomycin war vorwiegend (von 20 Stämmen bei 14) gegen Typ K : 2 wirkungsvoll. Chloramphenicol und Erythromycin waren ohne Rücksicht auf den Typ von mäßiger Wirkung. Stämme des Typs K : 28 und die Mehrzahl der sonstigen Typen waren chlor- und oxytetracyclinempfindlich, und zwar 26 von den 28 Stämmen des Typs K : 28 und 35 von den 54 zu sonstigen Typen gehörenden Stämmen. Zwischen dem Antibiogramm der aus dem Stuhl der enteralen Kranken und demjenigen der Kontrolle isolierten Klebsiella-Stämmen vom bekannten Serotyp konnten wir — mit Ausnahme des Typs K : 28 — keinen Unterschied beobachten. Von den aus der Gruppe der Kranken stammenden 23 Stämmen vom Typ K : 23 war das Antibiogramm von 22 übereinstimmend, während die 5 Stämme des Typs K : 28 der Kontrollgruppe zu vier verschiedenen Antibigrammtypen gehörten.

Die 189 in die Serotypen K : 1—30 nicht einreihbaren Klebsiella-Stämme gehörten zu 15 verschiedenen Antibigrammtypen. In der Gruppe der Kranken kamen die in den Antibigrammtypen 6, in der Kontrollgruppe aber die in den Antibigrammtypen 4 gehörenden Stämme in signifikant höherer Zahl vor (Tabelle IV).

Tabelle IV

Verteilung der Antibiogramm-Typen der serologisch nicht definierbaren (ND) Klebsiella-Stämme in der Kranken- und Kontrollgruppe

Antibiogramm Typ	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Aus dem Stuhl der Kranken isolierte Kl.-Stämme	4	—	1	10	11	35	—	9	8	10	14	3	1	1	2
Aus dem Stuhl der Kontrollen isolierte Kl.-Stämme	10	1	2	22	8	10	2	6	6	7	5	1	—	—	—

Von diesen Angaben und jenen in der Literatur [6, 7, 8] ausgehend, dürften wir auch mit dem häufigen Vorkommen von einigen Stämmen der Serotypen höherer Numerierung (K : 31—72) rechnen.

Klinische Beobachtungen. Von den in unserer Abteilung untersuchten 1014 Kranken litten 376 an gastroenteralen, 638 an extraenteralen Krankheiten. Klebsiella wurde in Verbindung mit 290 klinischen Fällen, in 28,5% des gesamten Krankengutes isoliert. Das Krankengut bestand aus 166 Enterocolitis-Kranken und 124 Kontrollen ohne enteralen Prozeß. Aus dem Stuhl von 33 enteralen Kranken isolierten wir gleichzeitig mit Klebsiella auch *E.*

coli dyspepsiae oder *Shigella*. Diese letzteren abgerechnet, wurde aus den 376 enteralen Kranken bei 133 (35,0%), in der Kontrollgruppe 19,4% *Klebsiella* ohne andere Pathogene isoliert. Die Angaben in bezug auf das enterale Krankengut sind in Tabelle V angeführt.

Tabelle V
Klebsiella ausscheidende enterale Kranken

Serotyp »K«	Zahl der enteralen Kranken	Prozentuale Verteilung des Krankengutes nach Typen	Zahl der Kranken mit		Verteilung der Infektionen		Gastro-Enterocolitis		
			<i>Klebsiella</i>	<i>Klebsiella</i> und bekannte Enteropathogene	Außenwelt	Krankenhaus	Schwer	Mittelschwer	Leicht
28	22	13,3	19	3	7 (3)	15	1	4	17
2	10	6,0	7	3	4 (1)	6 (2)	1	5	4
14	6	3,6	6	—	4	2	1	3	2
30	5	3,0	5	—	5	—	1	1	3
Sonstige Typen	23	13,9	17	6	18 (5)	5 (1)	2	2	19
ND-Typ	100	60,2	79	22	78 (14)	22 (8)	7	40	53
Insgesamt	166	100,0	133	34	116 (23)	50 (11)	13	55	98

NB — In Klammern ist die Zahl jener Fälle angegeben, bei denen auch bekannte enteropathogene Organismen isoliert wurden.

Wenn wir das enterale Krankengut nach Typen gruppieren, bildeten die Fälle mit Typ K : 28 einen bedeutenden Prozentsatz (13,3%). Auf die ausführliche Beschreibung derselben werden wir noch zurückkommen. Beachtenswert ist ferner das Zahlenverhältnis der die Typen K : 2, K : 14 und K : 30 ausscheidenden Kranken (6,0%, 3,6%, bzw. 3,0%). Die Mehrzahl der K : 28 und K : 2 Fälle bildeten diejenigen Kranken, bei denen die Infektion innerhalb des Krankenhauses erfolgte. Von den isolierten 33 bekannten enteropathogenen Keimen war *E. coli dyspepsiae* (O : 111, O : 55, O : 86) aus den Stuhlproben der Altersgruppe unter einem Jahr, *Shigella* (Flexner, Sonne) überwiegend aus denen der Altersgruppe über einem Lebensjahr zu züchten. Der Krankheitsprozeß lief, ohne Rücksicht auf den serologischen Typ, größtenteils unter dem Bilde einer leichten Gastroenterocolitis ab.

Wir teilten das aus 166 Enterocolitis-Fällen bestehende Krankengut auf Grund der enteralen Symptome in schwere, mittelschwere und leichte Krankheitsformen ein.

Schwere Gastroenteritis beobachteten wir bei 13 Kranken. Die Symptome waren Fieber zwischen 39 und 40° C, täglich mehrmaliges Erbrechen, täglich 6—10 flüssige, schleimige, eitrige, nicht selten blutige Stuhlentleerungen,

Schwäche, Eklampsie, toxischer Zustand. In sechs Fällen trat der enterale Vorgang zur extraenteralen Krankheitsform, meist zu Bronchopneumonie gesellt auf. Fünf Säuglinge wurden in atrophischen, dekomponiertem Zustand aufgenommen. Die Krankheit dauerte meistens 6—12 Tage. Der größere Teil der Patienten bestand aus Säuglingen (11 Kranken), zwei Kranke waren älter als ein Jahr. Nebst Klebsiella wurden aus dem Stuhl in drei Fällen enteropathogene Organismen (*E. coli* O : 111 B : 4 zwei Stämme, O : 86 B : 7 ein Stamm) isoliert.

Mittelschwere Gastroenterocolitis bestand bei 55 Kranken. Neben allgemeinen Symptomen standen täglich 6—8 dysenterieforme Stuhlentleerungen im Vordergrund. Ein ausschließlich enterales Krankheitsbild wurde bei 45 Patienten beobachtet, in den restlichen Fällen gesellten sich die Darmsymptome zu Otitis, Mastoiditis bzw. Bronchopneumonie. Nach einer Behandlung von 5—14 Tagen wurden die Patienten symptomfrei. Die Mehrzahl der Kranken bildeten auch in dieser Gruppe Säuglinge (I—II. Trimenon). Bekannte enterale Krankheitserreger mit Klebsiella gemischt ließen sich aus dem Stuhl von 14 Kranken züchten (*E. coli* O : 111 B : 4 11 Stämme, O : 55 B : 5, 1 Stamm, *Sh. flexneri* und *sonnei* je ein Stamm).

Leichte Enterocolitis kam in 98 Fällen vor. Bei Säuglingen äußerte sich die Krankheit in Temperaturen bis 38° C, Appetitlosigkeit, stagnierende Gewichtskurve, mitunter Erbrechen und die häufigere Stuhlentleerung. In der Altersgruppe über ein Jahr bildeten neben flüssigem, schleimigem, eitrigem, blutigem Stuhltyp auch die klinischen Symptome (beobachtbare Peristaltik, Tenesmus) ein auf Dysenterie hinweisendes Bild. Subfebrilität, diätetisch unbeeinflussbarer Durchfall und Darmbeschwerden lenkten in vielen Fällen die Aufmerksamkeit auf die infektiöse Ursache der Krankheit. Trotzdem konnten bei den 98 Kranken bloß in 16 Fällen enteropathogene Bakterien isoliert werden (*Sh. flexneri* in drei Fällen, *Sh. sonnei* in neun Fällen, *E. coli* O : 111 B : 4 in vier Fällen). 77 Kranken hatten nur enterale Beschwerden, 21 eine extraenterale Krankheit (Grippe, Pneumonie, Otitis) und auch Enterocolitis. Auf Antibiotikumbehandlung heilten die Kranken in 3—12 Tagen. Die überwiegende Mehrzahl der Kranken bestand aus Säuglingen unter sechs Monaten. Relativ groß war jedoch die Zahl der an dysenteriformer Enterocolitis leidenden Kranken im Alter über einem Jahr. Tabelle VI zeigt die Altersverteilung von den 166 an Gastroenteritis leidenden bzw. 124 enteral symptomfreien Kranken.

Als kausale Therapie verabreichten wir peroral Sulfonamid, Chloramphenicol, Chlortetracyclin, Neomycin, i. m. Sulfonamid, Streptomycin, Oxytetracyclin und Polymyxin B; bei schweren Zuständen, namentlich bei Säuglingen i. v. Tetracyclin-Präparate. Die symptomatische Behandlung bestand aus Ringer-Dextrose, Plasmainfusion, Mikrotransfusionen, Dosierung von Polyvitaminen und entsprechender Diät.

Tabelle VI

Verteilung der enteralen Kranken und der Kontrollen nach Alter

Enterale Kranken	0-3 Monate	4-6 Monate	7-9 Monate	10-12 Monate	über 1 Jahr	Insgesamt
Schwer	5	3	3	—	2	13
Mittelschwer	21	9	10	6	9	55
Leicht	21	12	9	10	46	98
Kontrollen	15	32	15	18	44	124

Die saisonmäßige Gestaltung der Zahl der an Enterocolitis Erkrankten mit einem positiven Klebsiellabefund zeigte eine Häufung im Frühjahr und im Herbst (Tabelle VII).

Tabelle VII

Monatliche Verteilung der Gastroenterocolitiden

(Auf Grund der anlässlich der ersten Untersuchung erfolgten Isolierung)

Typ	M o n a t e											
	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.	XII.
Klebsiella	2	4	4	13	20	10	11	4	17	35	9	4
Klebsiella + bekannte enteropath. Keime	—	—	—	7	4	3	3	2	1	12	1	—
Bekannte enteropath. Keime ..	4	8	17	38	32	18	16	4	19	43	17	5

Mit der Häufung der reinen Klebsiellafälle konnten wir in den Monaten April—Juni die Kulmination der *E. coli dyspepsiae* + Klebsiella, im Laufe der Monate September—November diejenige der Shigella + Klebsiella-Infektionen beobachten. Auf die erwähnten Frühjahrs- und Herbstperioden konzentrierte sich auch das Vorkommen der ausschließlich durch *E. coli dyspepsiae* bzw. Shigella verursachten Gastroenterocolitiden. Neben den bakteriologisch verifizierbaren Krankheitsfällen konnte bei zahlreichen Kranken trotz täglicher Beimpfung bloß Klebsiella, oder eine im Verhältnis zur normalen Darmflora relativ größere Menge von Klebsiella-Kolonien gefunden werden. Während der Krankheit war die Zahl der isolierten *E. coli* Kolonien auffallend klein; oft fehlten sie auch völlig. Auf das Aufhören des enteralen Prozesses lenkte auch die Rückkehr der Coli-Flora unsere Aufmerksamkeit. Die Symptome der während den Herbstmonaten behandelten Shigella negativen Kranken nahmen einen dysenterieformen Charakter an. Es sei erwähnt, daß es eben in Verbindung mit diesen Fällen gelang, den Serotyp K : 28 zu isolieren.

Wir haben auf das signifikant häufigere Vorkommen des Typs K : 28 im enteralen Krankenmaterial hingewiesen. Im Laufe der eingehenden Aufarbeitung des Krankenmaterials tauchte die Möglichkeit auf, daß die Stämme vom Typ K : 28 in den dysenteriformen Enterocolitiden eine ätiologische Rolle spielen dürfen. Dies schien auch die Tatsache zu bekräftigen, wonach von 22 Kranken 15 den Stamm in unserer Abteilung erwarben — bei ihrer Aufnahme

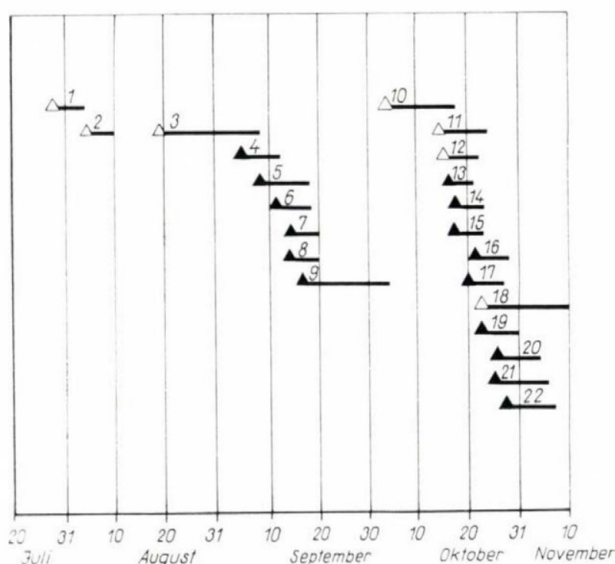


Abb. 1. Anstalts-Dyspepsieepidemie mit Klebsiella K : 28

△ Infektion äußeren Ursprungs

▲ Innerhalb des Krankenhauses erworbene Infektion

war der Stuhlbe fund Klebsiella negativ — und sich auch die enteralen Symptome erst nach der Aufnahme entwickelten. Den Stamm K : 28 haben wir am 28. VII. 1958 aus dem Stuhl eines von außerhalb des Krankenhauses aufgenommenen, an Enterocolitis leidenden Säuglings — der Stuhl enthielt keine bekannte enteropathogene Keime — zum erstenmal isoliert. Anschließend daran züchteten wir obigen Stamm im Laufe des Monats August aus dem Stuhl von weiteren zwei Kranken, die mit dyspeptischen Beschwerden aufgenommen wurden (Abb. 1).

In unserer Abteilung begannen die gehäuften Fälle von Dyspepsie mit den Symptomen des Kranken Nr. 4 mit Stamm K : 28 am 6. IX., und bis zum 18. IX. wurden davon 8 Säuglinge betroffen. Der Stamm wurde in den ersten zwei Wochen von Oktober aus den Stuhlproben von 3 neueren, mit dysenteriformer Enterocolitis zur Aufnahme gelangten Kranken, die sonst einen negativen Stuhlbe fund hatten, gezüchtet. Am 16. Oktober brach mit der Erkan-

kung des Kranken Nr. 13 eine zweite Hausepidemie aus, im Verlaufe dessen 9, hauptsächlich Kinder im Alter von 1—2 Jahren erkrankten. Auf diese Periode fiel auch die 3 Tage lang dauernde Enterocolitis der einen Krankenschwester mit Ausscheidung von K : 28. Aus den täglichen Stuhlproben ließen sich bloß bei 3 dyspeptischen Säuglingen, welche die Krankheit außerhalb des Krankenhauses erworben hatten, bekannte enteropathogene Keime züchten (je ein *Sh. flexneri*, *Sh. sonnei* und *E. coli* O : 111 B : 4).

Bei der Aufnahme wurde eine bakteriologische Stuhluntersuchung systematisch durchgeführt. Der Kranke konnte nur dann in die Abteilung gelangen, wenn keine enteropathogenen Organismen isoliert wurden. Im positiven Fall wurde der Patient in einen eigens für diesen Zweck reservierten Krankensaal untergebracht. Bei sonstigem Befund nahmen wir keine Isolierung der Kranken vor, welcher Umstand — trotz Einhaltung aller hygienischen Maßnahmen — die Verbreitung von Kreuzinfektionen und so des Stammes K : 28 ermöglichte. Der Krankheitsprozeß begann mit Fieber zwischen 38° und 39° C und mit flüssigem Stuhl, der dann einen schleimigen, eitrigen oft blutigen Charakter annahm. Das klinische Bild entsprach einer dysenteriformen Enterocolitis. 18 Kranken litten an rein enteralen Symptomen, 4 Kranken an sich zur Grippe bzw. Bronchopneumonie gesellter Enterocolitis.

Der im allgemeinen leicht ablaufende, 5—8 Tage dauernde Krankheitsprozeß reagierte gut auf die Behandlung. Eine schwere toxische Krankheitsform beobachteten wir in einem Fall, einen mittelschweren Zustand in 4 Fällen. Die mit einem extraenteralen Prozeß komplizierten Fälle beanspruchten eine Pflegezeit von 16—21 Tagen. Das Antibiotogramm der anlässlich der Epidemien isolierten Stämme war übereinstimmend.

Besprechung

Die Eruiierung der ätiologischen Faktoren der Darminfektionen unbekannter Herkunft im Säuglings- und Kindesalter ist eine komplizierte Aufgabe, umso mehr als neben den mit Hilfe der üblichen Züchtungsmethoden isolierten aeroben und anaeroben Bakterien mit dem Vorkommen und der pathogenen Rolle sonstiger Mikroorganismen (Protozoone, Pilze, Viren) gerechnet werden muß. Besonders die Beurteilung der Rolle jener Bakteriengruppen bildet eine Schwierigkeit, die im Darmkanal auch unter normalen Verhältnissen vorzufinden sind. Zu bewertbaren Schlüssen kann man daher nur durch die Analyse der vergleichenden Angaben der an größerem Spital- oder klinischem Krankengut oder bei Hausepidemien gewonnenen systematischen Untersuchungen gelangen.

Nach unseren Beobachtungen läßt sich *Klebsiella* aus den Stuhlproben der Kranken, im Verhältnis zur deren der Kontrollen, in größerem Prozentsatz isolieren. Demgegenüber ist das Verteilungsverhältnis der isolierten Serotypen

— abgesehen vom Typ K : 28 — sowohl in der Kranken- als auch in der Kontrollgruppe fast übereinstimmend. Ähnlich zu den untersuchten sonstigen Darmbakterien-Gruppen wird die Darmflora durch das relativ häufigere Vorkommen einiger Klebsiellaantigene (K : 28, K : 2, K : 14) charakterisiert. Die jetzt gefundenen häufigeren Serotypen unterschieden sich jedoch von den vorher nachgewiesenen Typen (K : 16, K : 11, K : 13). Die Häufigkeit der Typen ist wahrscheinlich je nach Krankenhaus, ja sogar je nach Säuglingsabteilung verschieden. Hierauf verweisen auch die Beobachtungen von ØRSKOV und Mitarb. [10].

Die Häufung der Gastroenterocolitiden mit Klebsiella »positivem« Befund ist meist in den Frühjahrs- und Herbstmonaten zu beobachten. Zweifellos weisen in diesen Perioden auch die *E. coli dyspepsiae* bzw. Shigella Infektionen eine größere Frequenz auf. Beachtenswert ist jedoch, daß der überwiegende Teil der in Reinkultur züchtbaren Klebsiella-Stämme eben in den erwähnten Zeitabschnitten zu finden ist. Die Züchtbarkeit der dominierenden Typen beschränkt sich ebenfalls auf die Frühjahrs- und Herbstmonate.

Im Zusammenhang mit den bakteriologisch nicht verifizierbaren Dyspepsien konnten wir uns bloß in bezug auf die eventuelle ätiologische Rolle des Serotyps K : 28 eine Aufklärung verschaffen, und zwar durch die, den Charakter einer Krankenhausepidemie annehmende Gestaltung der Infektionen. Die mit dem Erscheinen der enteralen Symptome parallel ausgeschiedenen, aus dem Stuhl in Reinkultur isolierbaren K : 28 Stämme, deren übereinstimmende Antibiotikumempfindlichkeit, ferner die auf die gezielte Therapie eintretende klinische Heilung und das Aufhören der Stammausscheidung unterstützten unsere Vorstellung bezüglich der ätiologischen Rolle des Typs K : 28. Die Rolle anderer Antigentypen läßt sich nicht so scharf abgrenzen, obwohl die Kreuzinfektion durch K : 2 und K : 14 in einigen Fällen als bewiesen betrachtet werden konnte.

Scheinbar schaffen die enteralen Grundkrankheiten, die in etwa 20% der Enterocolitiden nachweisbar sind, ferner der atrophische dekomponierte Zustand einzelner Kranken, durch die Verminderung der allgemeinen Resistenz eine günstige Grundlage für die Darminfektionen der Säuglinge.

Als Konklusion unserer Beobachtungen ließ sich Klebsiella Typ K : 28 mit den Hausepidemien in ätiologischen Zusammenhang bringen, und wenn es auch nicht gelang, eine entsprechende Zahl von Angaben zum Nachweis der pathogenetischen Rolle sonstiger Serotypen zu sammeln, so kann man unter Beachtung verschiedener Gesichtspunkte — in Einklang mit unseren früheren Feststellungen — die fakultativ enteropathogene Rolle der Klebsiella-Gruppe in den Gastroenterocolitiden des Säuglings- und Kindesalters als wahrscheinlich betrachten.

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INFLUENCE OF TEMPERATURE AND pH ON THE HAEMAGGLUTININATING ACTIVITY AND HAEMAGGLUTINATION-INHIBITION TEST OF ADENOVIRUSES

By

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Summary. The influence of temperature and pH on the agglutination of rat erythrocytes by adenovirus type 9 have been studied.

Out of four different temperatures ($+4^{\circ}\text{C}$, room temperature, 37°C in water bath and the same in incubator, and 40°C), $+4^{\circ}\text{C}$ and room temperature proved to be favourable. The same result was obtained with types 10 and 13.

In the pH range of 6.0 to 10.0, pH 9.0 was the most favourable and this was followed by pH 8.5, but the differences were too small to be of practical importance. The sensitivity to adenovirus haemagglutinin of the erythrocyte suspension did not decrease, during storage in the range from pH 7.0 to 9.0 it even increased.

The adenovirus haemagglutination-inhibition test was not influenced by the pH.

The discovery of direct haemagglutination (HA) by adenoviruses has provided a valuable test in both virus research and laboratory diagnostics. Out of the 28 adenovirus types discovered so far 26 have agglutinating properties [2]. The phenomenon can be inhibited by type-specific immune sera [3]. During adenovirus infections the HA-inhibiting antibodies appear in the serum earlier, attain considerably higher levels and are demonstrable for longer periods than the neutralizing and complement-fixing antibodies [4]. Consequently, the HA-inhibition (HI) test is preferable to the complement-fixation test [5] and, because of its simplicity, it can compete with the neutralization test. The HI test is certainly suitable for providing preliminary information and in certain cases appears to be more reliable than the neutralization test [2]. We have found the test well suitable as a routine diagnostic procedure [7].

In the present studies the influence of certain factors on the haemagglutinating activity of adenoviruses has been examined with types giving complete HA with rat erythrocytes [3]. The effect of the temperature and the pH was also studied.

Materials and methods

Viruses. Adenovirus strains belonging to types 9, 10, and 13 were propagated in Detroit-6 cell cultures adapted to rabbit serum [9]. The virus-infected cultures were frozen and thawed several times, and clarified by centrifugation. The virus suspensions thus obtained, which contained approximately 10^3 TCID₅₀ per ml, were kept at -20°C until used. Before the experiment a basal dilution of 1 : 4 was prepared.

Immune serum. Anti-adenovirus-9 serum was prepared in rabbits as described previously [10] and stored at -20°C . For HI tests a basal dilution of 1 : 8 was prepared.

Erythrocyte suspension. Three times washed erythrocytes of albino rats (*Rattus norvegicus*) were suspended in saline to which 1.0 per cent rabbit serum had been added to stabilize the HA reaction [3]. There was a considerable individual variation in the rats as to the degree of agglutinability of their erythrocytes by each of the three strains under study [12]. Erythrocyte suspensions were stored at $+4^{\circ}\text{C}$, at most for 48 hours.

Haemagglutination tests. Two parallel twofold dilution series were prepared, one starting with 1 : 8, the other with 1 : 12. TAKÁTSY's Microtitrator [11] was used. The sera were diluted in equal volumes of the erythrocyte suspension. The procedure was published in detail elsewhere [10]. Every titration was performed in three parallel rows and the last dilution causing agglutination of at least three-fourth of the erythrocytes (in the case of divergence, the arithmetic mean) was registered as titre. The results were read after 30 minutes, except for the cold titration ($+4^{\circ}\text{C}$) where sedimentation took at least 45 minutes.

HI tests. The virus was titrated immediately before the HI tests under identical conditions (equipment, temperature, pH); the saline used for the dilution of virus was always adjusted to the pH applied in the experiment. The serial serum dilutions were prepared with the micro-loop in the virus suspension as diluent. Each of the serum-virus mixtures contained 4 HA units of virus. The serum dilutions were 1 : 16, 1 : 24, 1 : 32, 1 : 48 etc. Every test was performed in three parallel rows. The virus-serum mixtures were kept at room temperature for 30 minutes. Subsequently, the erythrocyte suspension (1 per cent in saline with 1 per cent rabbit serum) was added; the results were read after an incubation of another 30 minutes, at the given temperature. As final titre, the highest dilution inhibiting the agglutination of more than 50 per cent of the added erythrocytes was registered.

Adjustment of pH. pH was measured by Clamann — Grahnert's MV 11 apparatus. The pH of the basal dilutions of serum and virus, of the saline, and of the erythrocyte suspensions, were adjusted to the desired value by adding 0.25 N NaOH or 0.25 N HCl.

Results

The HA tests were carried out at four different temperatures, viz. at $+4^{\circ}\text{C}$, room temperature, 37°C (parallel in the water bath and in the incubator), and in water bath at 40°C . In the first series of experiment the pH was measured, but not altered ("original pH", Table I). In these experiments the pH ranged from 7.0 to 7.5. The further experiments were performed at pH 5.0, 6.0, 7.0, 7.5, 8.0, 9.0 and 10.0. In certain cases the erythrocytes were destroyed by pH 5.0. The total number of experiments in this series was 186.

Table I shows that the titres were higher at $+4^{\circ}\text{C}$ and at room temperature than at 37°C or at 40°C . In the last two columns of Table I the geometric means of the $+4^{\circ}\text{C}$ and room-temperature values and of the 37°C and 40°C values, respectively, are presented. Similar experiments were carried out with adenovirus types 10 and 13, both of which belong to the same HA group as type 9 according to ROSEN's scheme [2] (Table II). The results are in conformance with those presented in Table I.

The $\log_2$ values of the titres were subjected to analysis of variance. The titres obtained at $+4^{\circ}\text{C}$ and at room temperature were compared to the titres obtained at 37°C and 40°C (Table III). Most of the differences proved to be significant statistically. The two nonsignificant differences (adenovirus 9 at pH 7.5 and 9.0) were near to significance.

Since, irrespective of the pH, the highest titres were obtained at $+4^{\circ}\text{C}$ and at room temperature, the experiments aimed at the determination of the optimum pH were carried out at these two temperatures.

Table I

Adenovirus type 9 titrated at various temperatures and pH
(geometric means of numerous parallel HA titrations)

pH	HA titres* at temperatures					Average	
	+4° C	room temperature (r.t.)	37° C		40° C	+4° C and r.t.	37° C and 40° C
			incubator	water bath			
"original"	124	113	93	96	86	118	91
6.0	108	92	75	75	65	100	72
7.0	106	103	77	83	76	105	79
7.5	111	96	90	94	82	103	89
8.0	121	111	88	93	81	116	87
9.0	126	109	91	86	83	118	86
10.0	118	118	98	97	88	118	94

* reciprocals

Table II

HA titres of adenovirus types at various temperatures

Adenovirus type	HA titres* at temperatures				40°C	Average	
	+4°C	room temperature (r.t.)	37°C			+4° C and r.t.	37° C and 40° C
			incubator	water bath			
9	124	113	93	96	86	118	91
10	256	278	215	215	183	267	204
13	32	30	24	18	19	31	20

* reciprocals

Table III

Analysis of variance for HA titres obtained at +4° C and room temperature, and those obtained at 37° C and 40° C

	Adenovirus type								
	9						10	13	
pH	6.0	7.0	7.5	8.0	9.0	10.0	original		
n	19	19	19	19	19	19	72	20	12
diff	0.48	0.41	0.22	0.41	0.45	0.32	0.38	0.39	0.62
t	3.116	2.752	1.760	2.998	3.490	1.959	3.791	2.564	2.497
P%	<1	<1	>5	<1	<0.1	>5	<0.1	<5	<5

The experiments of Series II a total of 480 experiments were carried out at 6 different pH values (6.0, 7.0, 7.5, 8.0, 9.0 and 10.0). Since the differences between the titres obtained at $+4^{\circ}\text{C}$ and those obtained at room temperature were negligible, the results were analysed together. The lowest titres were obtained at pH 6, then up to pH 9 the titres were increasing, whereas at pH 10 lower titres were obtained than at pH 9.

In order to find the most favourable pH, some experiments were carried out at pH 8.5. In this series (Series III, 132 experiments, Table IV) the pH values 7.0, 7.5, 8.0, 8.5, 9.0, and 10.0 were examined. In these experiments pH 9.0 was more favourable than pH 8.5.

Table IV

Geometric means of HA titres of adenovirus type 9 at various pH values

Series of experiment	HA titres* at pH						
	6.0	7.0	7.5	8.0	8.5	9.0	10.0
II	94	114	116	130	—	160	119
III	—	142	160	144	186	239	169

* reciprocals

A further analysis of the titres has shown that the titres obtained at pH 9.0 were significantly higher than those obtained at any of the other pH values tested (Table V). In spite of their mathematical significance, in view of the wide scattering the differences were of little practical importance.

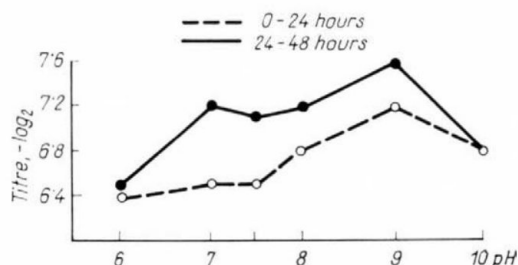
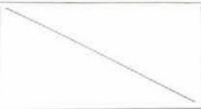


Fig. 1. HA titration of adenovirus suspensions with fresh and 24- to 48-hour-old erythrocyte suspensions. Average titres at pH 6.0–10.0

It was also examined how the HA titres are influenced by the age of the erythrocyte suspension (334 experiments). One- to 24-hour-old suspensions were compared to 24- to 48-hour-old ones. In Fig. 1 the titres expressed in $\log_2$ are plotted against pH. The divergence between the two curves was slight.

Table V
Analysis of variance for HA titres obtained at different values of pH (Adenovirus type 9)

pH	6.0	7.0	7.5	8.0	9.0	10.0	II
7.0		0.27 <5 2.148	0.30 <5 2.387	0.46 <0.1 3.660	0.76 <0.1 6.046	0.34 <1 2.705	6
7.5	0.17 >10 1.437		0.03 <5 0.239	0.19 >5 1.512	0.49 <0.1 3.898	0.07 >50 0.557	7
8.0	0.02 >80 0.169	-0.15 >20 1.268		0.16 >20 1.273	0.46 <0.1 3.660	0.04 >70 0.312	7.5
8.5	0.39 <0.1 3.297	0.22 >5 1.860	0.34 <1 2.874		0.30 <5 2.387	-0.12 >30 0.955	8
9.0	0.75 <0.1 6.340	0.58 <0.1 4.903	0.73 <0.1 6.171	0.36 <1 3.043		-0.42 <0.1 3.341	9
10.0	0.25 <5 2.113	0.08 >40 0.676	0.23 >5 1.944	-0.14 >20 1.183	-0.50 <0.1 4.227		10
III	7	7.5	8	8.5	9	10	pH

Explanation:

diff	t
P%	

- Notes: 1. $\text{diff} = \log_2 (\text{titre at higher pH}) - \log_2 (\text{titre at lower pH})$
 2. Standard deviation for the $\log_2$ values: $s = 0.886$
 3. Above the diagonal, Series II; below, Series III

Nevertheless, the differences in favour of the oldest suspension are well visible within the range from pH 7.0 to pH 9.0.

Finally, the influence of pH on the HI titres was examined at two temperatures and five pH values, in 210 experiments (Table VI). Appreciable divergences were not found.

Table VI

Geometric means of homologous HI titres of anti-adenovirus-9 serum at various pH values

pH	7.0	7.5	8.0	9.0	10.0
$-\log^2$ titre	12.1	11.9	12.1	12.1	12.4

Discussion

The first question was that of the most favourable temperature for adenovirus HA, with rat erythrocytes. Both $+4^\circ\text{C}$ and room temperature were found to provide the highest titres. This finding conforms to ROSEN's observations [1]. Although identical titres were obtained at these two temperatures, we recommend room temperature because at $+4^\circ\text{C}$ the sedimentation of erythrocytes is slower and in the case of large-scale experiments placing of plates properly in the refrigerator may cause difficulties. It should be noted that the present results are only valid for the technique used by us and for rat erythrocytes. We had to apply another method when glass tubes were used [10]. With monkey erythrocytes or other types of adenoviruses, higher temperatures seemed to be more favourable [12].

As regards the pH, the highest titres were obtained at pH 9.0. However the differences were so small that the use of that pH provides only little advantage in routine work. Some advantage may be expected at pH 9.0 in the case of freshly isolated strains, as here the HA titres are at the limit of detectability.

The pH of the erythrocyte suspensions was found to decrease during storage. Accordingly, the pH of stored erythrocyte suspensions should always be re-adjusted.

The question whether stored erythrocyte suspensions are suitable in the HA test for adenoviruses, was of interest because preparation of the suspension on the previous day would save time in the case of large-scale titrations. The fact that the stored erythrocytes were found to be somewhat more sensitive than the fresh ones has proved that storage for one or two days may be reasonable.

The HI titres of the sera were found to be independent of the pH since before every experiment the virus was titrated and strictly 4 HA units of virus were used in every test.

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ÉTUDES DES PROPRIÉTÉS DES ENZYMES PÉNICILLINASE ET PÉNICILLINE ACYLASE À L'OCCASION DE LEUR COEXISTENCE

par

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Résumé. Une souche du genre *Nocardia* non spécifiée produit à la fois les enzymes pénicillinase et pénicilline acylase. Tandis que l'action optimale de l'enzyme pénicilline acylase est située à pH 8,7, la pénicillinase du même origine montrait sa plus haute activité à pH 5,8. A cause de cette différence existantes entre les valeurs optimums des pH on peut déterminer l'activité de la pénicillinase en présence de celle d'acylase. L'endopénicillinase constitutive brute a agi sur le sel potassique de benzylpénicilline et sur l'acide 6-aminopénicillanique dans des mesures différentes. Les constantes de MICHAELIS (K_m) pour la benzylpénicilline étaient de $2,5 \times 10^{-5}$ M et pour l'acide 6-aminopénicillanique de $3,4 \times 10^{-3}$ M. Nous avons ensuite examiné les effets des divers composés sur la pénicillinase et sur la pénicilline acylase. Les sels sodiques d'acide benzoïque et d'acide sulphylique lesquels étaient déjà connus comme inhibiteurs de la pénicillinase étaient aptes à réduire l'activité de pénicillinase sans diminuer l'activité de la pénicilline acylase. L'effet semblable du pyrogallol était inconnu jusqu'à présent. L'acide phénylacétique et la quinine entravèrent l'action de la pénicillinase dans une faible mesure. L'acide phénylacétique trouble également l'action de l'acylase. Le degré de l'inhibition dépend de la concentration d'acide phénylacétique. La formaldéhyde exerce son influence sur l'acide 6-aminopénicillanique par un mécanisme chimique.

Une partie des microorganismes possède une activité enzymatique pénicilline acylase [1—4]. Cette enzyme en scindant la liaison $—CO—NH—$ de la molécule pénicilline produit l'acide 6-aminopénicillanique (le noyau de la pénicilline) et une acide organique qui désigne le type de pénicilline.

Récemment on a publié des observations sur ce fait que parallèlement à la formation de pénicilline acylase le même microorganisme peut former aussi l'enzyme pénicillinase [5]. Par l'effet de la pénicillinase la liaison $—CO—N=$ du noyau de la pénicilline s'hydrolyse et un produit biologique-ment inactif se forme.

Dans son expériences-ci nous avons étudié quelques propriétés de ces deux enzymes qu'influencent un substratum identique.

Partie expérimentale

Microorganisme. Les conditions de la culture. La préparation d'enzymes. Le microorganisme dont on s'est servi était une souche de *Nocardia* non spécifiée. La souche donnant une coloration jaune-orangée provient de la collection du DR. J. URI de L'Institute Pharmaceutique de l'Université de Debrecen. Nous avons cultivé le microorganisme dans un milieu liquide dont la composition est la suivante: caséine hydrolysée 2%; cornsteep liquor (50%) 0,1%; KH_2PO_4 0,28%; Na_2HPO_4 0,18%; $NaCl$ 0,20% dilués dans de l'eau distillée. Le pH avant stérilisation était de 7,0. La stérilisation a été faite à l'autoclave à une température de 120°C pendant 45 minutes. La culture a été accomplie à 28°C sur une table vibrante à 290 tours per min.

Après 48 heures de culture satisfaisante centrifugation à 8000 tours/min pendant 10 minutes. La masse des cellules provenant du culot a été lavée trois fois à l'eau distillée puis déshydratée par deux volumes d'acétone. Ensuite la masse des cellules desséchée, portant les enzymes pénicillinase et pénicilline acylase, a été homogénéisée et son poids sec a été déterminé. Pour les expériences nous avons employé cette préparation enzymatique. La pénicilline acylase brute était spécifique au sel potassique de benzylpénicilline.

Matériaux et abréviations. Le sel potassique de benzylpénicilline (PBP) avait 100%, l'acide 6-aminopénicillanique (6-APA) avait 96% et le sel potassique de l'acide phénylacétique (PhA) avait 96% de pureté.

Déterminations. La détermination manométrique de l'activité de la pénicillinase [6, 7] en présence de pénicilline acylase n'était pas utilisable parce que le bioxyde de carbon libéré qui aurait dû servir comme base pour les essais se fixe en réagissant avec l'acide 6-aminopénicillanique formée entre-temps [8, 9]. A sa place pour exprimer l'activité de la pénicillinase nous nous sommes servis de la différence entre la concentration initiale du substratum (PBP) et la concentration de l'acide 6-aminopénicillanique formée, plus la concentration finale du PBP. Les déterminations du PBP et 6-APA ont été exécutées par une méthode iodométrique [10]. Pour l'évaluation des unités internationales de benzylpénicilline en unités de poids de 6-APA nous avons utilisé la formule suivante:

$$6\text{-APA } \mu\text{g} = \frac{\text{unité internationale de PBP}}{2,7}$$

Résultats

En employant la préparation enzymatique on avait observé qu'en tout les cas il y a une disparité lorsqu'on compare la quantité de l'acide 6-aminopénicillanique formée comme produit de réaction, avec la quantité de benzylpénicilline disparue. Le déficit dépassa cette quantité théorique qui peut résulter de la dismutation chimique de benzylpénicilline ou bien de l'acide 6-aminopénicillanique dans les conditions d'expériences (Tableau I). Ce déficit en pénicilline indique la présence d'une enzyme pénicillinase agissant concurremment avec la pénicilline acylase. Cette enzyme même fut caractérisée comme endopénicillinase.

La valeur optimale du pH des enzymes penicilline acylase et pénicillinase. Selon les indications de la littérature la valeur optimale du pH de pénicillinase varie selon son origine et le substratum employé. Dans le cas de cette préparation enzymatique de *Nocardia* la détermination des valeurs optimales de pH a été rendue difficile par l'effet simultané de pénicilline acylase et pénicillinase sur un substratum identique dans une système de plusieurs composantes.

Pour que nous éliminions ce désavantage, pour une quantité donnée d'enzyme nous avons choisi les concentrations du substratum de sorte que la concentration initiale de la benzylpénicilline ne soit pas un facteur limitant l'activité de la pénicillinase, mais la quantité d'acide 6-aminopénicillanique formée pendant le temps de l'analyse soit négligeable.

L'optimum de pH de l'endopénicillinase de *Nocardia* était similaire (pH 5,8) pour chacun des deux substratums (c'est à dire pour la benzylpénicilline et l'acide 6-aminopénicillanique) (Figure 1). D'autre part il y avait une plus grande latitude sur la courbe du pH dans le cas du substratum benzylpénicilline.

Tableau I
La production d'acide 6-aminopénicillanique de benzylpénicilline

	Concentration initiale du PBP UI/ml	6-APA formée µg/ml	PBP titrée en retour UI/ml	Déficit de PBP UI/ml
Enzymes de <i>Nocardia</i> sp.	16000	1700	5410	6000

Conditions expérimentales: tampon de phosphate de pH 8,7; préparation enzymatique 1%. Incubation durant 10 heures à 28°C.

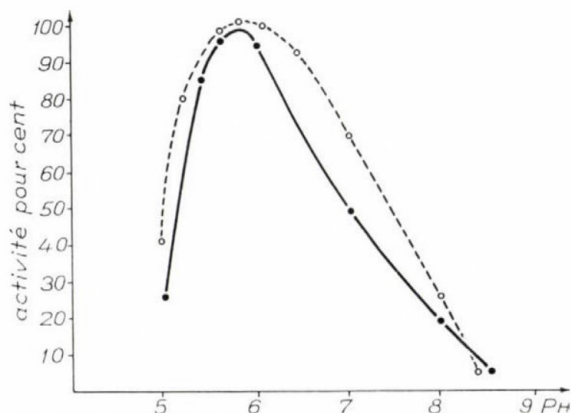


Fig. 1. Corrélation entre la valeur du pH et l'activité de la pénicilline de *Nocardia* en utilisant comme substratum le benzylpénicilline et l'acide 6-aminopénicillanique. Condition d'essai: PBP: 0,5 mg/ml, 6-APA: 0,5 mg/ml. Entre pH 5,0 et 6,0 tampon de potassium hydrogénéphthalate (0,1 M) — NaOH, entre 6,0—8,7 tampon de phosphate (après SÖRENSEN) sont utilisés. Incubation durant 2 heures à 28°C

○——○ : PBP; ●——● : 6-APA

L'activité de la pénicilline en pH 8,7 qui est la valeur optimale de pH de pénicilline acylase avait seulement 5% d'efficacité originale. Au regard de valeur optimale de pH de pénicilline acylase (pH 8,7), il en résulte que les optimums de pH de ces deux enzymes ont une différence significative (Tableau II).

La vitesse relative d'hydrolyse de la chaîne fermée de β-lactame et les constantes de MICHAELIS pour les substratums benzylpénicilline et l'acide 6-aminopénicillanique. Les essais de détermination ont été exécutés à un pH 5,8 à 37°C. Les vitesses initiales ont été déterminées entre 0,5 mg—5,0 mg/ml pour le 6-APA et 0,05—5,0 mg/ml pour le PBP. Nous avons obtenu les valeurs de $V_{\max}$ pour le 6-APA 0,012 mg min⁻¹ ml⁻¹, pour le PBP 0,19 mg min⁻¹ ml⁻¹. Après la correction avec les poids moléculaires le rapport d'hydrolyse était donc

$$\frac{0.19}{0.012} \times \frac{216}{372} = 9,18$$

Tableau II

Les valeurs optimales du pH de la pénicilline acylase et de la pénicillinase

	L'optimum du pH de		
	pénicilline acylase	pénicillinase pour 6-APA	pénicillinase pour benzylpénicilline ou phénoxy-méthyl-pénicilline
<i>Nocardia</i> sp.	8,7	5,8	5,8
<i>Str. lavendulae</i> *			
pour phénoxy-méthyl-pénicilline	9,0	—	environ 7,0
pénicillinase de <i>staphylococcus</i> *	—	5,6	6,5—7,0
pénicillinase de <i>staphylococcus</i> **	—	—	5,8
pénicillinase de <i>B. cereus</i> *	—	5,5	7,0

* BATCHELOR *et al.*, 1961 [11], ** NOVICK, 1962 [12].

Tableau III

L'effet de diverses pénicillinases sur la benzylpénicilline et l'acide 6-aminopénicillanique

	K_m pour PBP	K_m pour 6-APA
<i>Nocardia</i> sp.	$2,5 \times 10^{-5}$ M	$3,4 \times 10^{-3}$ M
pénicillinase de <i>B. cereus</i>	$4,3 \times 10^{-6}$ M ¹	$3,0 \times 10^{-3}$ M ²
pénicillinase de <i>staphylococcus</i>	$3,4 \times 10^{-4}$ M ²	$3,0 \times 10^{-3}$ M ²
pénicillinase de <i>staphylococcus</i>	$2,5 \times 10^{-6}$ M ³	—

¹ POLLOCK, 1960 [14], ² BATCHELOR *et al.*, 1961 [11], ³ NOVICK, 1962 [12].

Appliquant la méthode de LINEWEAVER et BURK [13] les valeurs de K_m obtenues sont représentées au Tableau III. Il s'ensuit que les valeurs de K_m de la benzylpénicilline et de l'acide 6-aminopénicillanique varient entre des larges limites dépendante de l'origine de la pénicillinase. Nous attirons l'attention sur identité intime des valeurs du K_m en cas du *Nocardia* et du *B. cereus* pénicillinases.

L'effet des inhibiteurs sur l'activité de la pénicillinase. Pour d'entraver la forte activité de la pénicillinase l'application des inhibiteurs paraît bien motivée. En cherchant les inhibiteurs convenables nous devons prendre en considération la présence simultanée de la pénicilline acylase. Nous avons caractérisé la condition d'utilisation d'un inhibiteur par son effet sur la pénicillinase en sauvegardant l'intégrité de l'activité de la pénicilline acylase. Dans cette expérience nous avons utilisé l'acide 6-aminopénicillanique comme substratum pour pénicillinase. Pour exprimer le degré de l'inhibition sur la pénicillinase

nous nous sommes servis d'un rapport (A) que nous définîmes par la formule suivante:

$$A = \frac{\text{la vitesse de l'hydrolyse en présence d'inhibiteur}}{\text{la vitesse de l'hydrolyse sans inhibiteur}}$$

En conséquence $A \rightarrow 0$ exprimait le degré de l'inhibition. Il ressort du Tableau IV que les inhibiteurs traditionnels de la pénicillinase, les sels sodiques d'acide benzoïque et d'acide sulphanylique étaient aptes à affaiblir remarquablement l'activité de la pénicillinase. Un effet similaire était décelé aussi en ce qui concerne le pyrogallol dont ce caractère n'était pas connu jusqu'à présent. L'effet antipénicillinasique de l'acide phénylacétique est digne d'attention parceque c'est l'un de composés libérés par l'activité de l'acylase. L'effet du formaldéhyde sur la benzylpénicilline et l'acide 6-aminopénicillanique avait une nature chimique.

Tableau IV

L'effet des inhibiteurs sur l'endopénicillinase d'une souche du genre Nocardia

Composé et concentration	A
Arsenure de sodium $10^{-2} M$	1,00
Acétate d'iode de sodium $10^{-2} M$	0,98
Cyanure de potassium $10^{-2} M$	1,00
Fluorure de sodium $10^{-2} M$	1,00
Formaldéhyde $10^{-2} M$	>1,00
Phénylacétate de potassium $10^{-1} M$	0,80
Benzoate de sodium $10^{-2} M$	0,30
Pyrogallol $10^{-2} M$	0,10
Sulphanylate de sodium $10^{-2} M$	0,35
Quinine chlorhydrique $2.10^{-2} M$	0,72
Éther de pétrole 3%	1,00
Amylacétate 1%	1,00
Dioxane 1%	1,00
Acétone 10%	1,00

Condition d'essai: préparation enzymatique à 1% dans un tampon de monopotassium-hydrogénephthalate — NaOH, pH 5,8; 1000 $\mu\text{g/ml}$ de 6-APA; 2 heures d'incubation à 37°C. Contrôle: pas d'inhibiteur.

L'effet des substances antipénicillinasiques sur la pénicilline acylase. Nous avons examiné comment des composés, qui inhibent l'activité de la pénicillinase, agissent parallèlement sur la pénicilline acylase. Ces essais ont été effectués à la valeur optimale de pH de la pénicilline acylase. L'action antipénicillina-

sique des composés était contrôlable à répétition par l'écart entre les quantités d'acide 6-aminopénicillanique formée, plus benzylpénicilline titrée en retour et la concentration initiale du substratum. L'action inhibitrice des substrates sur la pénicilline acylase était indiquée par l'abaissement de quantité absolue d'acide 6-aminopénicillanique formée. Il résulte du Tableau V que les inhibiteurs à l'exception du phénylacétate de potassium n'exercent pas une influence sur la formation de l'acide 6-aminopénicillanique ou bien sur l'action de la pénicilline acylase. Le sel potassique de l'acide phénylacétique réduit le rendement de l'acide 6-aminopénicillanique.

Tableau V

L'effet des inhibiteurs enzymatiques sur l'activité de la pénicilline acylase et la pénicillinase

	PBP titrée en retour UI/ml	6-APA formée $\mu\text{g/ml}$	différence en PBP UI/ml
Enzyme dénaturée	15650	0	350
Contrôle	4500	1700	6910
Acétate d'iodure de sodium $10^{-2} M$	4704	1720	6645
Quinine chlorhydrique $2 \times 10^{-2} M$	4800	2100	5530
Sulphanylate de sodium $10^{-2} M$	6350	2660	2448
Benzoate de sodium $10^{-2} M$	6450	2904	1710
Pyrogallol $10^{-2} M$	5900	3230	1379
Phénylacétate de potassium $10^{-1} M$	6700	1340	5682

Conditions d'essai : 16000 UI/ml de PBP; préparation enzymatique à 1%; tampon de phosphate de pH 8,7. Incubation durant 10 heures à 28°C.

L'influence du sel potassique de l'acide phénylacétique sur l'activité de la pénicilline acylase. Pour nous rendre compte si le sel potassique de l'acide phénylacétique agit directement sur la molécule de l'acide 6-aminopénicillanique ou bien il influence l'activité de la pénicilline acylase nous avons examiné la vitesse de décomposition d'une quantité connue d'acide 6-aminopénicillanique en présence du sel potassique de l'acide phénylacétique appliqué à des concentrations différentes. Il a été devenu évidente que même $8 \times 10^{-1} M$ du sel potassique de l'acide phénylacétique n'hydrolysait pas la chaîne fermée du β -lactame. En revanche la concentration croissante du sel potassique de l'acide phénylacétique exerçait une influence sur l'action de la pénicilline acylase. L'évolution de la quantité de benzylpénicilline résiduelle indiquait que l'effet inhibitif du phénylacétate de potassium sur la pénicillinase s'accroît parallèlement à sa concentration (Figure 2). L'acide 6-aminopénicillanique même en concentration appréciable (216.000 $\mu\text{g/ml}$) n'a pas d'effet pondrable sur l'activité de l'enzyme acylase.

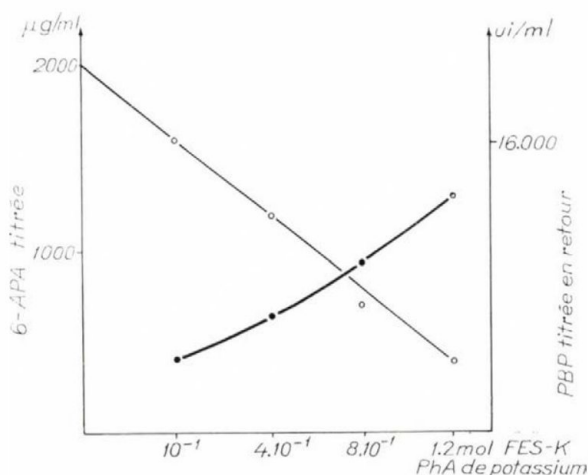


Fig. 2. L'effet de phénylacétate de potassium sur l'activité de la pénicilline acylase de *Nocardia* sp. Condition d'essai: préparation enzymatique de *Nocardia* sp. à 1%. Tampon de phosphate de pH 8,7 (après SØRENSEN). PBP 16000 UI/ml. Phénylacétate de potassium en concentrations croissantes. Incubation durant 10 heures à 28°C

○ — ○ : concentration de 6-APA après l'incubation
 ● — ● : concentration de PBP après l'incubation

Discussion

Par l'effet scindant de la pénicilline acylase [15] sur la liaison $-\text{CO}-\text{NH}-$ du substratum benzylpénicilline (a), une molécule d'acide 6-aminopénicillanique (b) et une molécule d'acide phénylacétique (c) se forment. L'action hydrolytique de la pénicillinase sur la chaîne fermée de β -lactame aboutit à la formation d'acide pénicilloinique (d), de benzylpénicilline et d'acide 6-aminopénicilloinique (e), d'acide 6-aminopénicillanique. Par suite de la décarboxylation spontanée de l'acide pénicilloinique et la réaction du bioxyde de carbone avec l'acide 6-aminopénicillanique plusieurs composés se forment qui peuvent influencer la vitesse de production de 6-APA (produit principal) puis son extractibilité du mélange de réaction (Figure 3).

A la valeur optimale du pH de la pénicillinase l'activité de la pénicilline acylase est négligeable. Par conséquent à ce pH on peut étudier l'influence de la pénicillinase sur le benzylpénicilline et l'acide 6-aminopénicillanique. On peut expliquer les différences parmi les valeurs K_m d'une part avec la dissemblances stéréochimiques des molécules de benzylpénicilline et d'acide 6-aminopénicillanique et d'autre part avec les conditions de perméabilité des parois cellulaires de certains microorganismes.

La différence entre les valeurs optimales du pH des deux enzymes nous donne la possibilité par l'augmentation du pH en milieu alcalin de diminuer l'effet malencontreux de la pénicillinase. L'utilisation de certains inhibiteurs de la pénicillinase rend possible de diminuer encore d'avantage l'activité de l'endopénicilline provenant de la souche *Nocardia*.

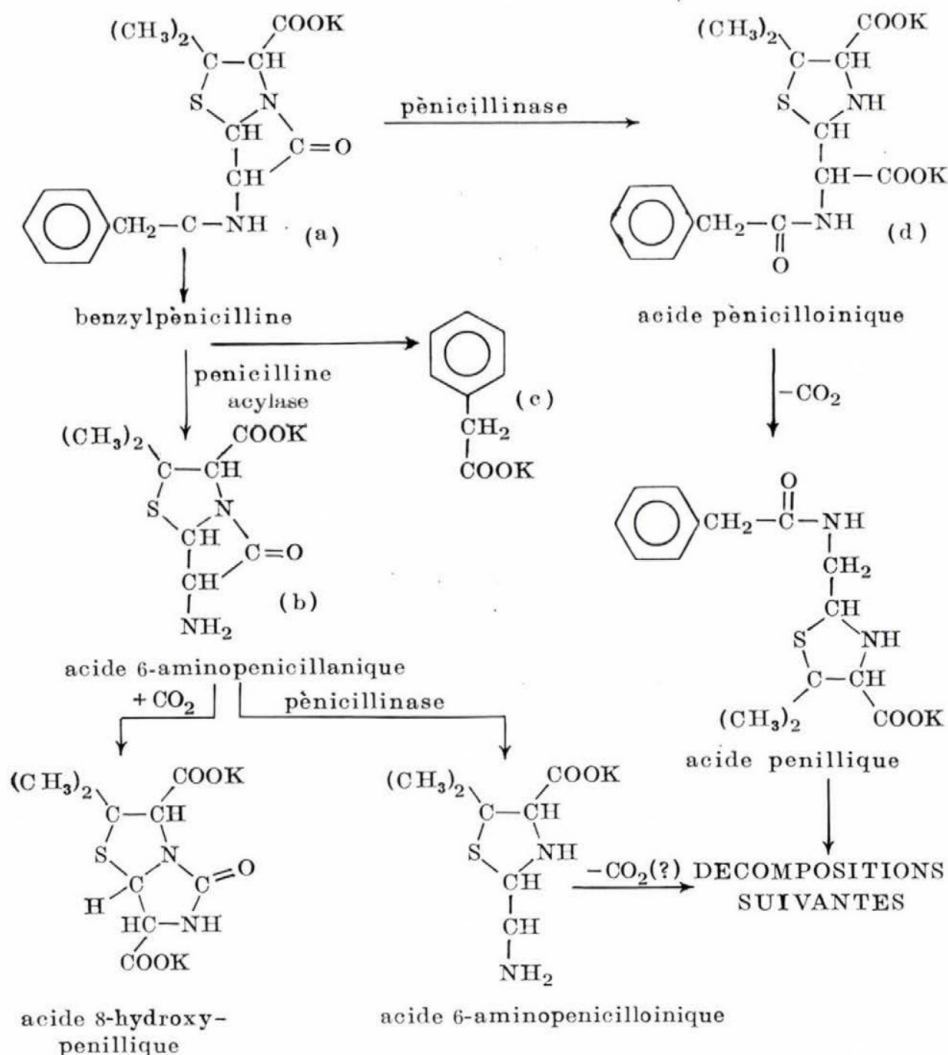


Fig. 3. La décomposition de benzylpénicilline en présence simultanée de pénicilline acylase et de pénicilline (pour détails cf. le texte)

Nous avons confirmé les observations de REID, FELTON et PITTOFF [16] et de CARR [17] sur l'effet antipénicillinasique des sels sodiques de l'acide benzoïque et de l'acide sulphylique et le résultat similaire de VÁCZI et URI [18] obtenu par la quinine. D'autre part nous n'avons pas pu répéter les expériences de PÉRAULT [19] et de McQUARRIE et LIEBMAN [20] sur l'effet inhibitif de l'acétone de l'amylacétate et du dioxane sur la pénicilline. Nous décrivons le pyrogallol et l'acide phénylacétique comme inhibiteurs nouveaux de la pénicilline.

La force perturbatrice de l'acide phénylacétique, formée par l'action de la pénicilline acylase sur la vitesse d'hydrolyse de la liaison $-\text{CO}-\text{NH}-$ résulte de deux facteurs: 1° dans le milieu s'acidifiant constamment avec la diminution de l'activité de la pénicilline acylase l'activité de la pénicillinase s'accroît; 2° l'acide phénylacétique, ou bien son sel potassique, libérés pendant l'hydrolyse, même en cas de compensation continue du pH à la valeur 8,7 déploiera graduellement un effet inhibiteur.

Les expériences ci-dessus nous montrent qu'il est utile d'étudier l'activité de pénicillinase que présentent occasionnellement des microorganismes ayant une activité de pénicilline acylase. Pour cette analyse un essai sur pH 5,8—6,0 avec l'acide 6-aminopénicillanique comme substratum semble convenable. On peut déterminer la concentration initiale puis finale du substratum à l'aide d'un titrage iodométrique. La même essai peut aussi indiquer la présence de pénicillinase dans le produit intermédiaire au cours de la purification de la pénicilline acylase.

L'analyse des effets de la pénicilline acylase et de la pénicillinase lors de leur coexistence, offre des possibilités pour la détection de leur spécificité sur les diverses pénicillines. L'analyse cinétique des actions enzymatiques fournit une base à l'étude des systèmes enzymes — substratums à plusieurs composants ou deux ou bien plusieurs enzymes agissent simultanément sur un substratum.

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STUDIES ON THE NUCLEIC ACID CONTENT OF STREPTOMYCES STRAINS

By

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Summary. The nucleic acid composition in different stages of growth of two *Streptomyces griseus* strains with short and of long vegetative phase, respectively, has been examined. The investigations revealed a possible explanation for the high degree of variation of the values found by other authors. The influence of the inoculum's amount upon the nucleic acid composition of the culture is shown. The difficulties in the evaluation of the biochemical study of streptomycetes is discussed.

The nucleic acid metabolism of streptomycetes has been investigated by several authors [2—5, 7, 9]. As different methods have been applied, the results cannot reliably be compared. The described data scatter considerably even with one and the same strain. The unusual variation in the nucleic acid content may be due to the mycelial heterogeneity of *Streptomyces* cultures, namely, to an alteration in the proportion of vegetative and reproductive elements. The purpose of the present experiments was to determine whether the high variation in nucleic acid content is a property of certain *Streptomyces* strains or some kind of correlation exists between the degree of the heterogeneity of mycelium and nucleic acid composition. The relative proportion of vegetative and reproductive elements was estimated by cytomorphological examinations [12]. For the experiments, two *Streptomyces griseus* variants, the long vegetative phase strain 52—1 and the short vegetative phase strain 45H were used [10]. The former was a streptomycin-producing, the latter a streptomycin non-producing mutant.

Materials and methods

The *Str. griseus* strains were cultivated on soya agar slants for 10 days. The cultures were suspended in saline by shaking with glass beads and inoculated into 100 ml Difco broth distributed in 500 ml Erlenmeyer flasks. The cultures were shaken at 28° C horizontally on a shaker operating at 230 r.p.m. and 5 cm stroke length. After 24, 48 or 96 hours the cultures were centrifuged and the mycelium was washed three times in saline. Fractionation of mycelium and spore suspension was performed as described by SCHMIDT and THANNHAUSER [8]. In this manner a cold trichloroacetic acid-extractable fraction containing mainly the sugar esters was obtained. After removing the lipids by extraction with alcohol-ether mixture, the suspensions were incubated in *N* KOH for 18 hours at 36° C. The amount of total nucleic acid was determined from this fraction. Then the DNA was precipitated with acid, and the supernatant containing the RNA, was decanted. DNA was extracted with hot trichloroacetic acid. The phosphorus content of the fractions was determined by the method of MARTLAND and ROBISON

[6]. The dry weight of the mycelium was determined as described by BARABÁS, SZABÓ and VÁLYI-NAGY [1]. The results were expressed in μg phosphorus/mg dry material. The data were calculated from the average of two experiments performed on two different occasions. Each experiment included three parallel observations.

Results

In the first experiments the nucleic acid content of the cultures was found to vary with different fermentations. As all the other experimental conditions were uniform, variations in the amount of inoculum were thought to be responsible for the differences. Therefore the phosphorus contents of the fractions prepared as described above were examined in two cultures started from inocula differing in one order of magnitude. In Tables I and II it may be seen that when different numbers of spores had been inoculated, the same strain varied more definitely as to nucleic acid distribution than two different cultures started from inocula containing equal numbers of spores. The nucleic acid values were higher with small than with large inocula. The nucleic acid content and composition of the mycelium depended on the age of the culture. The highest values were found in 24 hour cultures. The values of the nucleic acid fractions greatly decreased at 96 hours, with small inocula this tendency was observed as soon as after 48 hours' cultivation. The difference between the fractions was the highest after 24 hours with both small and large inocula. Of the nucleic acid compounds RNA-P is primarily responsible for the difference. In cultures seeded with equal numbers of spores, the RNA and DNA content of the two different strains did not differ significantly after 24, 48 and 96 hours. However, the DNA content of the short life cycle culture after 96 hours cultivation was double that in the long life cycle organism. In the Tables the RNA/DNA proportions are also shown. As growth proceeded, the RNA/DNA proportion decreased slightly in 52-1 cultures seeded with large inocula, and definitely in those inoculated with small numbers of spores. In contrast, with large inocula the RNA/DNA value decreased in strain 45H, but with small inocula it showed no alteration.

Fig. 1 indicates that the dry weight content of the whole culture differed most definitely after 24 hours of cultivation. The RNA and DNA values for the whole culture still increased in cultures started from small inocula, at the same period when the corresponding values showed no further increase in large inoculum cultures.

As shown in Table III, a difference was revealed in the phosphorus content of the spores of the two strains. The RNA-P and DNA-P values for the spores of strain 45H were higher than the same values for strain 52-1. With the exception of the mycelium from shaken cultures of strain 52-1 seeded with small inoculum, the spore DNA-P values were not identical with the corresponding 96 hour values in mycelial cultures.

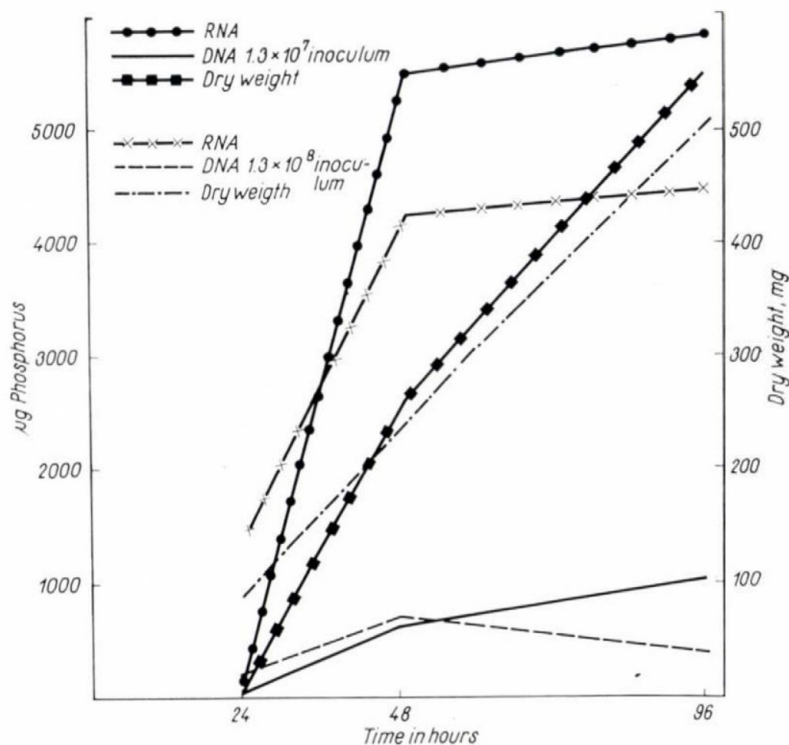


Fig. 1. Total RNA and DNA content in *Str. griseus* No. 52-1 cultures started from small and large inocula

Table I

Phosphorus values obtained in *Streptomyces griseus* 45H cultures started from small and large inocula

	Inoculum, no. of spores	24 hr.	48 hr.	96 hr.
Total P	1.3×10^8	31.5	27.0	15.1
	1.3×10^7	39.9	33.3	19.7
TCA-P	1.3×10^8	6.6	4.1	3.5
	1.3×10^7	—	5.3	3.9
Total nucleic acid P	1.3×10^8	23.6	22.8	11.4
	1.3×10^7	39.3	28.0	15.6
RNA-P	1.3×10^8	20.9	18.9	7.8
	1.3×10^7	32.5	24.0	12.9
DNA-P	1.3×10^8	2.7	4.0	3.5
	1.3×10^7	7.3	4.4	2.7
RNA/DNA P	1.3×10^8	7.0	4.0	2.0
	1.3×10^7	4.0	5.0	5.0

Table II

Phosphorus values obtained in Streptomyces griseus 52-1 cultures started from small and large inocula

	Inoculum no. of spores	24 hr.	48 hr.	96 hr.
Total P	1.3×10^8	31.6	34.8	13.9
	1.3×10^7	44.6	35.5	19.5
TCA-P	1.3×10^8	11.6	13.2	3.7
	1.3×10^7	—	12.2	6.4
Total nucleic acid P	1.3×10^8	20.0	21.8	10.1
	1.3×10^7	44.4	23.5	12.9
RNA-P	1.3×10^8	17.6	17.4	8.8
	1.3×10^7	40.0	21.3	10.6
DNA-P	1.3×10^8	2.4	2.9	1.1
	1.3×10^7	4.4	2.4	1.9
RNA/DNA-P	1.3×10^8	7.0	6.0	8.0
	1.3×10^7	9.0	8.0	5.0

Table III

Phosphorus values in spores of Str. griseus strains 52-1 and 45H

	Strain	
	45H	52-1
TCA-P	18.7	6.5
Total nucleic acid P	28.2	10.1
RNA-P	22.9	7.7
DNA-P	4.7	1.9

Discussion

The growing mycelium in submerged cultures, of *Streptomyces* strains passes through a vegetative, then a subsequent reproductive phase. The vegetative phase is characterized by a rapid growth. In the reproductive phase the growth ceases and various reproductive forms as spores, chlamydospores and fragments of hyphae appear. The biochemical characterization of these phases is desirable both theoretically and practically. The subsequent appearance of vegetative and reproductive forms is indicative of the differentiation of the microorganism. On the other hand, some kind of relationship exists between the growth phases and the

intensity of antibiotic production [3]. Examination of the nucleic acid content of cultures revealed that as regards phosphorus distribution, the hyphae differed only in young cultures seeded with small inocula. If it is assumed that the rapidly growing mycelium is characterized by a high nucleic acid value and RNA excess ($\text{RNA}:\text{DNA} > 5$) and that findings characteristic of reproductive forms (lower nucleic acid values and $\text{RNA}:\text{DNA} = 5$) are obtained with the spores, it may be concluded that the cultures contained a mixture of vegetative and reproductive forms. Young cultures started from small inocula, up to 24 hours of age, are somewhat exceptional, as in these the vegetative growth phase predominates.

As attempts at isolating various parts of the mycelium in amounts suitable for chemical analysis have failed, the heterogeneity of individual hyphae could only be assumed.

The hyphae in 24, 48 and 96 hour cultures could not be differentiated on the basis of the $\text{RNA}:\text{DNA}$ or nucleic acid: dry weight ratios.

From the experiments two main conclusions may be drawn. In order to obtain reproducible results, for the biochemical investigation of streptomycetes the culture should be started from a standardized inoculum. Biochemical processes characteristic of the vegetative and reproductive phases run simultaneously and, accordingly, the experiments yield the resultant of the two processes. Our experiments have shown that the heterogeneity of the cultures may present exceptional difficulties in the biochemical studies on streptomycetes.

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DIFFERENTIATION OF ENTERAL BACTERIA BY THE ALKALINE REACTION

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Summary. Enteral bacteria can be cultivated in NaCl containing aqueous peptone solution with acid pH and free of carbohydrates as well as of any other common carbon or energy sources. The alkaline by-products of the replication process give rise to pH-shifts in the alkaline direction, demonstrable by the colour change of the indicator. In the cultures of certain bacteria, an alkaline reaction occurs with a rapidity and regularity uncommon with other species. With an appropriate choice of medium and cultivation conditions, the family Enterobacteriaceae might be divided into several groups on the basis of the alkaline reactions presented. For this purpose the use of 5 peptone media is suggested, which differ in hydrogen ion concentration only, having pH values 5.4, 4.7, 4.5, 4.3 and 4.1, respectively. From the results obtained by examining a total of 1423 bacterium strains with the method, the following conclusions have been drawn. The peptone medium with the highest pH value was rendered alkaline by every strain tested. On the basis of the alkalizing capacity in the other 4 media, enteral bacteria may be divided into groups of low, medium or high alkalizing activity. To the first group belong *Shigellae*, *Proteus* and *Providencia*, to the second *E. coli*, *Citrobacter*, *Cloaca* and *S. typhi*, while to the third *Klebsiellae*, numerous *Salmonellae*, *Arizona*, and presumably also *Serratia*.

The microorganisms belonging to the family Enterobacteriaceae are capable of alkalizing the NaCl-containing aqueous peptone medium. The present studies were based on this property of enteral bacteria.

Materials and methods

Strains. The majority of the *Shigella* strains used was isolated from routine samples submitted in the previous year, while the minority belonged to our strain collection. The strains were identified with up-to-date methods [2, 5, 6]. Several strains of enteral bacteria were supplied from the stock of the Bacteriological Department and Phage Laboratory of our Institute. The cultures grown on DORSET's medium or agar slant were stored in the refrigerator. For the examinations, 18 to 24 hour subcultures were used.

Cultivation. The effect of environmental conditions on the alkalizing activity of strains was studied in preliminary experiments, using a fluid medium containing 0.5 per cent NaCl and 1 per cent peptone. After inoculation the media were incubated for 5 days at 37° C and the occurrence of colour changes was checked daily. The lack of a colour change was regarded as a negative result, while a transitory colour shade as indicative of neutral pH ($\pm$). In the alkaline region low (+, ++), or high (+++, ++++) values were differentiated. By the above method the influence of the following factors was examined.

Of the peptone preparations examined (Bacto Proteose, Bacto Casamino, Witte, Richter and Bacto Difco peptone), Bacto peptone seemed to be the most suitable for our purposes, since it allowed clearly to differentiate the alkalizing capacity of strains. For the same reason bromocresol purple in 0.0024 per cent concentration was used as an indicator. The alkalizing activity was found to be markedly influenced by the amount of culture medium used as well as by the original pH value and free surface of the latter. The germ count of the inoculum had also a decisive role. Accordingly, reliable results are obtained only when the test is performed with utmost care. A correct adjustment of the pH after sterilization is of primary importance.

The glassware coming into contact with the medium should be scrupulously clean. Scratched and opaque glassware must not be used for alkaline reaction tests.

Massive inocula containing about 10^{10} germs elicit a colour change immediately after suspension in the peptone medium. Presumably, with the agar culture used as inoculum, alkaline valencies are transferred into the peptone water. An immediate colour change can be eliminated if prior to inoculation the bacteria are washed 3 times in sterile saline. Nevertheless, the alkaline reaction remains positive despite the above washing procedure if the usual amount, i.e. 10^7 germs are used as inoculum.

Routine method. On the basis of preliminary experiments the following method was applied for testing the alkalizing activity of enteral bacteria. In one litre of distilled water 5 g NaCl and 10 g Bacto peptone were dissolved during heating. The solution was boiled for 5 or 6 minutes. During cooling 12 ml of BCP solution (0.10 g bromocresol purple and 2.5 ml N/5 NaOH dissolved in 47.5 ml distilled water) were added. The peptone solution thus prepared was divided into 5 equal parts and with N/10 HCl or N/10 NaOH the pH of the parts was adjusted to pH 3.9, 4.1, 4.3, 4.5 and 5.0, respectively, having considered the pH-rise generally occurring after sterilisation. Then the media were passed through filter paper and the first 4 lots were sterilized with steam for 30 minutes, while the last — pH 5.0 — lot in the autoclave for 15 minutes at 120° C. After sterilisation the pH of the lots was controlled whether they have the appropriate values, i.e. 4.1, 4.3, 4.5, 4.7 and 5.4, respectively.

Five ml amounts of the sterilized media were distributed into 16 by 160 mm tubes under sterile conditions. As an inoculum pin-head quantities of 24 hour slant cultures of the bacterium strains were used. During incubation for 3 days at 37° C the occurrence of a colour change was checked daily. Alkalinity is easily recognized by the development of a blue or lilac shade. Closer observation is necessary only when the pH-shift in the alkaline direction is limited; this is indicated by a greyish colour. Yellow, yellowish or whitish cultures were considered negative.

Accordingly, the reaction may remain negative throughout incubation or turn positive in 1 to 3 days. The findings are in fair accordance with the designation recently used for evaluation [7]. Thus, alkaline reactions occurring after 1 day of incubation are designated “+”, those developed after 2 days “(+)” and no reaction throughout with “—”. As seen in Table II, *d* and *x* results were also found.

The intensity of the lilac colour was neglected in routine examinations. However, the intensity of colour reaction runs usually parallel to the alkalizing activity of the culture.

Results

Alkalizing activity of enteral bacteria. Under appropriate conditions bacteria belonging to the family Enterobacteriaceae can be cultivated in carbohydrate-free acid peptone medium which is gradually rendered alkaline by the by-products of bacterium replication (Fig. 1).

On prolonged incubation further pH shifts in the alkaline direction occur and the value of potential OH ions reaches the peak earlier than the actual concentration of OH ions (Table I).

To demonstrate the alkalizing activity of bacteria on solid media, a medium is prepared of peptone water with 2 per cent agar, with the pH adjusted e.g. to pH 6.0. Around the colonies a halo with blurred margins occurs, displaying colour changes in dependence of the indicator used. Since the alkaline substances diffuse into the medium, the colour of the agar layer also changes. In stab cultures the colour reaction reaches different depths, with an indistinct lower border.

Use of the alkaline reaction for biochemical testing of Enterobacteriaceae. The method described above might be used for the differentiation of enteral bacteria. Data concerning 1423 strains of enteral bacteria are summarized

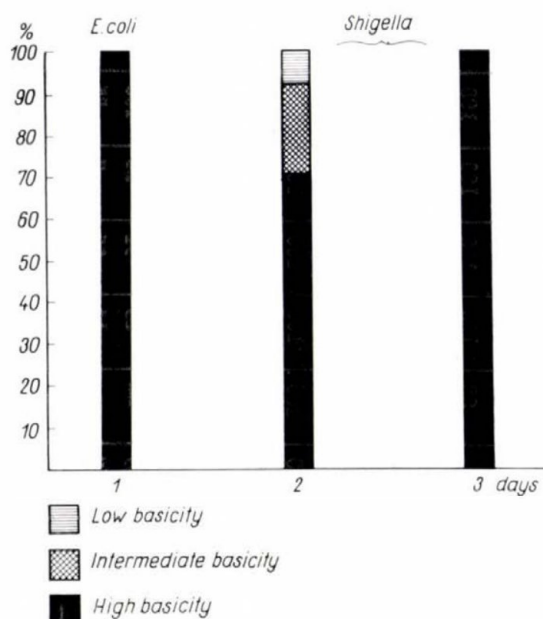


Fig. 1. Basicity of peptone cultures of *E. coli* and *Shigella* strains after 1 and 2 days incubation. Medium: 1 per cent Proteose peptone solution, pH 5.7; Inoculum: pin-head amount; Cultivation conditions: aerobic; Strains: 100 *E. coli* and shigellae each

Table I

Changes of basicity during prolonged incubation of *E. coli* cultures grown in peptone medium
5 ml pH 5.7 Bacto peptone medium, pinhead inoculum, aerobic cultivation

Time of incubation (days)	The amount of N/10 HCl required to neutralize 5 ml of cultures (ml)	pH
1	1.9	6.7
2	3.5	7.3
3	4.8	7.4
4	5.3	7.5
5	6.2	7.6
6	6.8	7.7
7	7.0	7.8
8	7.2	8.0
9	7.2	8.1
10	7.2	8.2

Table II
Alkaline reactions of enteral bacteria

Strains	No of strains	Percentage of positive cultures at pH														
		4.1			4.3			4.5			4.7			5.4		
		Days														
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
<i>E. coli dyspepsiae</i> ^a	160	7	58	85	55	96	98	81	100	100	91	100	100	100	100	100
strains O, K and H	268	10	70	91	24	89	97	50	92	99	57	97	100	85	100	100
0124	88	—	—	66	—	59	86	—	92	100	6	96	100	64	100	100
<i>Klebsiella</i>	22	18	100	100	68	100	100	73	100	100	82	100	100	100	100	100
<i>Citrobacter</i>	22	—	68	93	—	95	100	68	100	100	86	100	100	100	100	100
<i>Cloaca</i>	17	88	93	94	94	100	100	94	100	100	94	100	100	100	100	100
<i>Serratia</i>	6	33	100	100	67	100	100	100	100	100	100	100	100	100	100	100
<i>Salmonella typhi</i>	69	—	3	63	13	39	97	48	88	100	58	94	100	94	100	100
<i>S. paratyphi</i> ^b	254	74	100	100	90	100	100	93	100	100	100	100	100	100	100	100
Arizona	19	47	80	100	53	100	100	74	100	100	79	100	100	100	100	100
<i>Shigella dysent.</i> 1—10	25	—	—	—	—	—	28	—	24	25	—	25	48	18	44	88 ^c
<i>Sh. flexneri</i>																
1, 2, 4, 5, 6	198	—	—	—	—	—	3	—	5	27	—	9	40	21	82	100
3	33	—	—	—	—	—	6	—	21	79	—	25	91	24	100	100
<i>Sh. boydii</i> 1—15	72	—	—	—	—	—	13	—	4	42	—	14	55	7	55	100
<i>Sh. sonnei</i>	83	—	—	15	—	—	79	—	52	96	—	49	97	29	81	100
<i>Proteus</i>																
<i>vulgaris</i>	20	—	—	—	—	—	—	—	—	25	—	15	40	80	100	100
<i>mirabilis</i>	19	—	—	—	—	—	16	—	16	58	—	42	79	79	100	100
<i>morganii</i>	21	—	—	—	—	—	—	—	—	—	—	—	76	95	100	100
<i>rettgeri</i>	12	—	—	—	—	—	—	—	—	—	—	—	—	—	54	100
Providence	15	—	—	—	—	—	—	—	33	40	20	40	60	33	67	100

^a) 026, 055, 086, 0111, 0119, 0125, 0126, 0127, 0128

^b) *S. paratyphi* B, *derby*, *typhi murium*, *stanleyville*, *kunzerdorfi*, *bovis moribificans*, *paha*, *duesseldorf*, *enteritidis*, *anatum*, *cerro*

^c) within additional 2 days 100 per cent

in Table II. Certain strains were available in a limited number only; their data are presented to give a complete survey.

According to our results, on the basis of the alkaline reaction 2 groups of enteral bacteria can be sharply differentiated. One of them, designated by us as *low basicity group*, consisting of *Shigella*, *Providencia* and *Proteus* is — with the exception of a few *Sh. sonnei* cultures — characterized by a negative alkaline reaction in both pH 4.1 and 4.3 peptone medium, after 3 and 2 days of incubation, respectively. The *high basicity group* of *Salmonella* (except *S. typhi*), *Klebsiella*, *Arizona* and, presumably, also *Serratia*, renders the pH 4.1 peptone medium alkaline in 3 days, while the pH 4.3 medium in 2 days of incubation. On the basis of their behaviour in acid peptone medium the low and high basicity groups of enteral bacteria are clearly distinguishable. With regard to their activity in the above medium, the rest of *Enterobacteriaceae*, i.e. *E. coli*, *Citrobacter*, *Cloaca* and *S. typhi*, might be regarded of *medium basicity*. In this group the differentiation of cultures from low basicity ones is possible only when they elicit colour change in pH 4.1 and pH 4.3 media within 3 and 2 days, respectively, and in both pH 4.5 and 4.7 peptone medium within 1 day of incubation. From high basicity cultures medium basicity ones can be distinguished by their eventual negative alkaline reaction in pH 4.1 medium within 3, while in pH 4.3 and 4.5 media within 2 days of incubation. The alkaline reaction of serologically or antigenically related strains may be different, as demonstrated in Table II for the alkaline reactions of *Sh. sonnei*, *P. rettgeri* and *S. typhi* strains.

In the pH 5.4 peptone medium an alkaline reaction was presented by every strain examined, although certain *Shigella* strains rendered the medium alkaline after 5 days of incubation only. Our results support the hypothesis that an alkaline reaction is characteristic of the family *Enterobacteriaceae*.

The mechanism of the alkaline reaction. The role of bacterial multiplication in base production is obvious since multiplication must always precede the production of base. This is further supported by the fact that peptone water becomes turbid soon after inoculation, a colour change occurs only when a certain degree of turbidity has been reached (Tables III and IV).

A delay of the alkaline reaction occurs when multiplication is protracted. The low multiplication rate being an inherent property of certain bacterial strains, it occurs not only in peptone water but also in complete medium, i.e. pH 7.2 bouillon (Table V).

According to the recommended technique, large doses of inoculum are used with which different substances from the precultures might also be transferred. In order to eliminate these factors, the transfers made from 4 *E. coli* and 6 *Shigella* cultures positive in the previous test were effected with 1 small loopful each of bacteria washed 3 times in sterile saline and suspended in 1 ml peptone water. Washing did not interfere with the development of an

Table III

*Alkaline reaction and live germ count*1 ml pH 4.5 peptone medium; inoculum $\frac{1}{3}$ loop

Time of incubation	Strains					
	781. <i>E. coli</i> 0111		57. <i>Sh. flexneri</i> 6		72. <i>Sh. flexneri</i> 3	
	basicity	germ count per ml	basicity	germ count per ml	basicity	germ count per ml
0 hour	—	2×10^7	—	4×10^7	—	6×10^7
4 hours	—	1×10^7	—	2×10^7	—	1×10^7
1 day	++++	5×10^8	—	6×10^7	—	4×10^6
2 days	++++	8×10^8	—	5×10^7	—	2×10^6
3 days	++++	9×10^8	++++	2×10^8	—	2×10^7
4 days	++++	6×10^8	++++	4×10^8	±	6×10^7
5 days	++++	7×10^8	++++	3×10^8	±	8×10^7
7 days	++++	5×10^8	++++	2×10^8	±	1×10^8
8 days	++++	7×10^8	++++	2×10^8	+	2×10^8

Table IV

Alkaline reaction and live germ count

5 ml pH 4.5 peptone medium; pin head inoculum

Incubation time	30 216 <i>E. coli</i> K : 62		30 218 <i>E. coli</i> K : 66	
	basicity	germ count per ml	basicity	germ count per ml
0 hour	—	3×10^7	—	2×10^7
4 hours	—	4×10^5	—	8×10^4
1 day	—	2×10^8	—	7×10^5
2 days	++++	5×10^8	—	2×10^8
3 days	++++	4×10^8	+++	4×10^8

alkaline reaction after incubation. Serial transfers to pH 5.4 peptone medium were also made, by using as inoculum $\frac{1}{3}$ loopful of the first agar subculture. Further passages were performed with 1 loopful of the peptone culture. Passage experiments were made with 15 *E. coli*, 10 *Sh. flexneri* and 5 *S. typhi murium* strains, of which 7 *E. coli* strains could be carried through 8 and 2 *Sh. flexneri* 3 strains through 5 passages when the experiment was discontinued. The adaptation of *S. typhi murium* was less successful; only 1 strain could be maintained in 3 subcultures. As a control 1 loopful of 24 hour bouillon cultures of the strains was transferred to the respective peptone medium simultaneously

Table V

Multiplication of E. coli strains of lower and higher basicity in bouillon

5 ml pH 7.2 medium; pin-head inoculum

Incubation time	Live germ count per ml	
	30 216 <i>E. coli</i> K : 62 high activity	30 218 <i>E. coli</i> K : 66 low activity
0 hour	2×10^7	2×10^7
4 hours	1×10^8	2×10^7
1 day	5×10^8	5×10^8
2 days	5×10^8	6×10^8
3 days	2×10^8	5×10^8

with the last passage. Multiplication and an alkaline reaction was shown by 4 of the 7 *E. coli*, and 1 of the 2 *Shigella*, strains. The different behaviour of the remaining 1 *Shigella* and 3 *E. coli* strains might be explained by adaptive enzyme synthesis.

A certain parallelism could be observed between the results of the alkaline and lysine decarboxylase reactions. Since in acid media bacteria are producing decarboxylase, for the basic character of cultures the amines formed as a result of decarboxylase activity are probably responsible. The ninhydrin test of CARLQUIST [1], performed with the chloroform-extract of bacteria from the positive strain was negative. Detailed studies are now being made to elucidate the biochemical processes involved in the alkaline reaction; it now appears to be based on the desamination of amino acids. The results of these experiments will be published elsewhere.

Discussion

Among the biochemical methods used for the identification of enteral bacteria, the recently developed decarboxylase tests [1, 8, 9] have proved to be useful in practice. However, for MØLLER's test a special peptone preparation is needed, and the necessary centrifugation makes it difficult to use CARLQUIST's method for mass examinations. For the alkaline reaction test yielding results fairly similar to the above methods, no special reagents are required and its technique is simple.

As shown by the data in Table II, an alkaline reaction in itself is unsatisfactory for the identification of enteral bacteria, while together with other biochemical tests it is a useful means of differential diagnosis. *E.g.* by their alkaline reaction even *E. coli* and *Shigella* or *E. coli* and *Salmonella* strains may be differentiated despite the medium basicity of *E. coli*. Cultures presenting

an alkaline reaction in pH 4.1 and pH 4.3 peptone water within 1 or 2 days or in pH 4.5 and pH 4.7 one within 1 day — like the majority of *E. coli* strains — might practically be excluded from the *Shigella* group. Except for *S. typhi* and *S. paratyphi A*, all the *Salmonella* strains examined induced an alkaline reaction in pH 4.1, 4.3 and 4.5 peptone medium after 2 days, and in the pH 4.7 medium after 1 day, of incubation, while the majority of *E. coli* strains remained negative under similar conditions. Differentiation of other groups of enteral bacteria is also possible. For the comparison of low and high basicity ones a single pH 4.1 peptone culture is sufficient.

The peptone media used in the present experiments did not contain carbohydrate or other common carbon and energy sources. Nevertheless, at acid pH the enteral bacteria are capable of multiplying in these media if conditions are not artificially rendered unfavourable. Consequently, enteral bacteria may utilize amino acids not only as nitrogen but also as carbon and energy sources even in acid media. This observation is supported by the positive results gained with inocula prepared of washed bacteria. The results of several serial passages, however, suggest that for enteral bacteria not requiring growth-enhancing factors, carbohydrate-free acid peptone water is a satisfactory medium.

The alkaline reaction must not be regarded as indicative of the extent of multiplication because the latter is not necessarily succeeded by the former. According to our routine procedure, cultures are observed for 3 days; nevertheless in part of them a colour change occurs on further incubation only. Generally, alkaline compounds may be formed without being indicated by a colour change because they do not necessarily reach the concentration required to elicit the respective pH-shift. The reason for this might be either the low concentration of alkaline compounds or the high change-over zone of the indicator used, e.g. of phenolphthalein.

Finally, the test is negative when the strain is unable to multiply in peptone water either because of the insufficient amount of inoculum used or owing to inadequate conditions, such as a high acidity of the medium. In one of our experiments for instance the pH was still acid 5 days after inoculation and when checked at 0 hour, 4 hours and 1, 2 and 3 days after inoculation the germ count of the culture was 6×10^7 , 4×10^5 , 1×10^3 , 2×10 and 0, respectively.

It ought to be mentioned that from older peptone medium cultures variants with different colony morphology could frequently be isolated.

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DEMONSTRATION OF THE SWINE-FEVER VIRUS IN TISSUE CULTURE BY IMMUNOFLOUORESCENCE

By

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Summary. Swine-fever virus was propagated in embryonic pig-kidney cell cultures and the virus antigen was demonstrated by the indirect fluorescent antibody technique. Trypsinized suspension of renal epithelial cells was distributed in Legroux flasks, on the bottom of which small slides had been placed. The cultures were infected with the wild virus of swine fever and, after variable periods of incubation, treated with rabbit immune serum prepared against the lapinized strain of swine-fever virus. (The serum was titrated by the agar diffusion precipitation test.) Subsequently, the preparations were treated with anti-rabbit-gamma-globulin serum produced in goat and labelled with fluorescein isothiocyanate, and examined under the fluorescence microscope.

An intensive cytoplasmic fluorescence was observed in contrast with the control preparations showing no characteristic fluorescence. The cytoplasmic fluorescence was associated with the antigen of the swine-fever virus, which was thus visualized by the indirect fluorescence method.

Demonstration of intracellular viral antigen by the fluorescent antibody technique is of special importance when virus multiplication is not accompanied by specific cytopathic changes and appropriate serological tests are lacking. A good example of this is the swine-fever virus. Although knowledge concerning this virus has been increasing since 60 years [1], it is still rather incomplete.

The propagation of the virus in porcine tissue cultures has been solved [2-6], but its cytopathic effect appears only under special conditions [7-9]. Consequently, inoculation of susceptible pigs was the only method available to demonstrate multiplication of the virus.

In the present study it was attempted to apply the fluorescent antibody technique in visualizing the swine-fever virus in pig-kidney cell cultures.

Materials and methods

Tissue culture. Renal epithelial cells of the pig embryo [10] obtained by trypsinization were suspended in Hanks' balanced solution. In 5 ml suspension 4×10^6 cells were placed in each of Legroux flasks containing eight slides of 3×1 cm. As growth medium 15 ml Hanks' solution containing 0.5 per cent lactalbumin hydrolysate and 2 per cent bovine serum were added. After three days of incubation the medium was changed for Parker's medium 199 containing 2 per cent bovine serum and the change of medium was repeated on every third day. At least two flask cultures were prepared from every cell suspension; one was inoculated with virus and the other remained as control. The cultures were incubated at 37°C.

Virus. Tissue cultures were infected with "Swine-Fever Virus" ("Phylaxia" State Institute for Serum and Vaccine Production) consisting of defibrinated blood taken at the peak of fever from pigs artificially infected with the virus. Preservative was not added.

Inoculation of cultures. After 7 to 8 days of incubation the medium was poured off carefully and one drop of the virus was dripped on each of the small slides, which were then

allowed to stand at room temperature. After 30 minutes 15 ml maintenance solution was added to each of the flasks and incubation was continued. Slides were removed for microscopical examination after 4, 24, 48, and 72 hours.

Immune serum. Apparently healthy rabbits were immunized with "lapinized swine-fever vaccine" (Phylaxia) consisting of lyophilized blood and spleen homogenate of rabbits inoculated with the lapinized strain of swine-fever virus and bled during the subsequent period of fever [11]. The preparation was diluted as prescribed by the producing firm and 2 ml were injected into the thigh muscle of each of two rabbits. Inoculation was repeated at five-day intervals. After the third intramuscular injection, eleven doses were given intravenously. Antibodies could not be detected by the agar diffusion test after the 10th dose; they appeared, however, after the 12th injection. For the 15th injection the vaccine was diluted threefold less than prescribed and 10 ml was injected intravenously. The rabbits were killed by bleeding 8 days thereafter. The sera were filtered through Seitz EK pad and titrated by the agar-diffusion precipitation test.

Anti-Aujeszky and anti-herpes sera were prepared by immunizing rabbits with Aujeszky's virus propagated in chick embryonic fibroblast cultures, and with herpes simplex virus propagated in HeLa cells respectively. To prepare anti-polio-1 serum, *M. rhesus* monkeys were immunized with virus propagated in rhesus kidney cultures.

Agar-diffusion precipitation test. The original test for the detection of swine-fever virus [12] was carried out in a modified form [13, 14]. To agar purified with CaCl_2 , methyl orange and thymersol were added in dilutions 3 : 100 000 and 1 : 10 000, respectively. The thawed agar was poured on slides and, after wells were made and the materials were placed in the wells, the slides were incubated at 37°C to accelerate reaction. The immune serum produced against the lapinized virus gave a positive reaction with the swine-fever virus up to the dilution of 1 : 512.

The immunofluorescent method. The indirect method [15] was found to be appropriate. The fluorescein-isothiocyanate-labelled anti-rabbit globulin was prepared in goats, as published elsewhere [16], and applied according to the classical method [17]. Before staining, the slides taken out of the flasks under sterile conditions were washed three times in PBS and dried at 37°C for an hour. After fixation in acetone for 10 minutes the slides were dried again for 30 minutes. Then nonlabelled, 1 : 10-diluted anti-lapinized-virus rabbit serum was dripped on the preparations at 37°C for 10 minutes and these were washed with three changes of PBS for 10 minutes. Subsequently, the 1 : 10-diluted fluorescein-isocyanate-labelled anti-rabbit goat globulin was added and the preparations were kept in the wet box for 30 minutes. Finally, the preparations were thoroughly washed with three or four changes of PBS for 30 minutes and sealed with PBS in 1 : 10-diluted glycerol.

Microscope and microphotos. A Zeiss microscope equipped with cardioid condensor and HBO-50 high-pressure mercury-vapour lamp and combined BG 3/2 and GG9/OG1 filters were used. To prepare microphotos, a Leitz "Ortholux" fluorescence microscope was applied with HBO-200 illumination. Agfacolor UK (16-DIN) films were used. Exposure time was 8 minutes.

Results

No fluorescence was demonstrated 4 hours after inoculation of cultures. The infected cultures appeared as dark as the control preparations (Fig. 1).

After 24 hours diffuse accumulation of a mass showing greenish-yellow fluorescence was visible in the cytoplasm of about one-third of the cells covering the slides (Fig. 2). Most of the cells showing cytoplasmic fluorescence formed small groups, which appeared as bright foci consisting of 4 or 5 neighbouring cells (Fig. 3). The nucleus including nucleolus remained dark. The contours of most of the cells were unaltered, only a few cells were rounded off or detached from the glass. Owing to this, the cultures showed reticular areas with a few rounded cells in the gaps of the network. The latter showed a bright cytoplasmic fluorescence.



Fig. 1. Uninfected pig-kidney epithelial cell culture (10-day-old) treated with anti-swinefever rabbit serum and fluorescein-isothiocyanate-labelled anti-rabbit-globulin. Magnification, $\times 400$

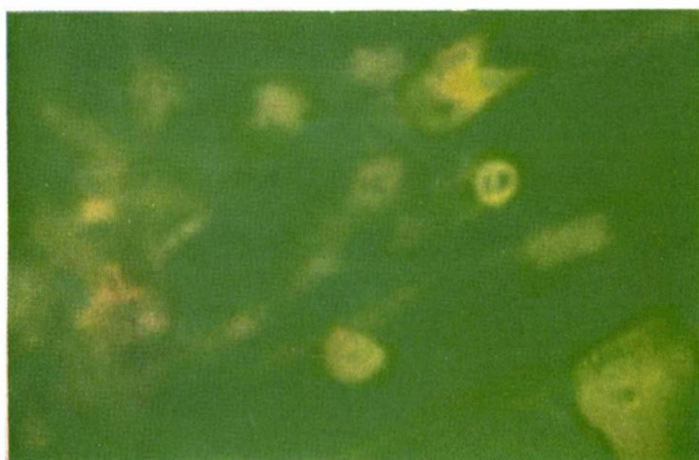


Fig. 2. Nine-day-old culture 24 hours after infection with the wild strain of swine-fever virus. Cytoplasmic fluorescence in about one-third of the cells. Magnification, $\times 400$

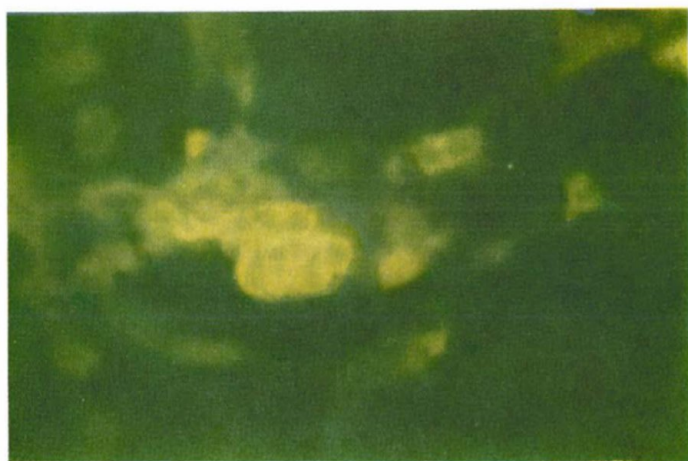


Fig. 3. Ten-day-old culture 48 hours after infection. Extensive cytoplasmic fluorescence, cell contours are hardly visible. Magnification, $\times 400$

By the 48th hour the cytoplasmic fluorescence was brighter and more extensive (++++); some groups of cells were so bright that the cell was hardly recognizable.

At 72 hours almost every cell showed a diffuse, strong cytoplasmic fluorescence. In general, the cells were unaltered morphologically.

Control preparations. Uninfected cultures of the same age prepared from the same cell suspension in the same manner served as controls. In these preparations no fluorescence was observed, except the pale, uniformly weak autofluorescence of the cell. This was obviously different from the specific fluorescence visible in the infected preparations, both in colour and intensity.

Swine-fever-virus-infected preparations treated with the anti-Aujesky, the anti-herpes or the anti-polio immune sera served as further controls. These, too, were examined 4, 24, 48, and 72 hours after inoculation. Neither of these preparations showed a fluorescence exceeding the autofluorescence shown by the uninfected controls.

Parallel with the immunofluorescence studies every component involved in the immune reaction as well as the nutrient media removed from the cultures were subjected to the agar-diffusion test. The results are summarized in Table I.

Table I

Agar-diffusion precipitation tests with the tissular compounds used in the immunofluorescence test

Antigen	Serum	
	anti-swine-fever**	normal rabbit
Pancreas suspension from pig with swine fever*	positive	negative
Swine-fever virus "Phylaxia"	positive	negative
Nutrient fluid of a cell culture infected with "Phylaxia" swine fever virus	positive	negative
Normal pig tissues (spleen, liver, kidney, lymph node)	negative	negative
Nutrient fluid of uninfected tissue culture	negative	negative

* According to MANSI's procedure [14].

** Prepared to the lapinized virus.

Discussion

The susceptibility to swine-fever virus of primary monolayer cultures of the pig embryo has already been reported [18, 19]. In the present experiments cell outgrowth was satisfactory in both the infected and the uninfected cultures, showing that the employed media and procedures were adequate.

In accordance with literary data [7, 8, 18], cytopathic changes did not appear in the present experiments, *i.e.* the propagation of the virus itself did not solve the problem of demonstration of the virus and did not provide

a suitable method for detailed research, *viz.* propagation of the virus in tissue cultures did not make unnecessary the inoculation of pigs. The agar diffusion test, the only *in vitro* test available [12], has widely been employed, especially in its recent modification [13, 14]; however, according to our own data, its fiducial does not exceed 65 per cent [20]. Besides, its specificity has not been fully proved. Recently, tests based on virus exaltation [21] and on interference [22] have been published, but these tests do not demonstrate the virus itself, thus provide only indirect evidence of the presence of the virus in tissue cultures.

The object of the present studies was to develop a specific method of detection of the swine-fever virus. The fluorescent antibody technique proved to be suitable for this purpose. The indirect test was chosen and, to avoid species-specific fixations, rabbit immune sera were used.

The lapinized strain had originated from the ROVAC strain of the Lederle Laboratories, it was adapted to the rabbit by alternative passages [23] and underwent 245 rabbit passages before incorporated in the commercial vaccine [24]. The strain is immunologically indistinguishable from the wild virus of porcine origin [23, 11], as confirmed by the present results obtained by the agar diffusion test.

For further studies it seems to be desirable to produce immune sera more effective than that prepared by us in rabbits with the lapinized virus.

The specificity of the fluorescence observed in the present studies has been proven by the lack of fluorescence when cultures infected with swine-fever virus were treated with normal rabbit serum or with sera obtained from rabbits immunized with other viruses, such as Aujeszky-disease virus, herpes simplex virus, poliovirus, as well as in uninfected preparations treated with anti-swine-fever serum.

The lack of fluorescence in the four-hour cultures in contrast to the bright fluorescence after 24 hours shows that the fluorescence cannot be attributed to the mere presence of the virus, but virus multiplication also took place.

It can be concluded that we succeeded in demonstrating by means of the fluorescent antibody technique the multiplication of swine-fever virus in primary epithelial cultures of embryonic pig kidney. It is hoped that the further use of this technique will provide more detailed data concerning the mechanism of swine-fever infection.

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STUDIES ON INTRATYPIC VARIANTS OF ECHOVIRUSES

I. SEPARATION OF HAEMAGGLUTINATING AND NON-HAEMAGGLUTINATING VIRUS PARTICLES FROM ECHOVIRUS TYPE 6

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Summary. Three out of 10 strains of echovirus type 6 isolated from routine material were only found to possess haemagglutinating activity. The behaviour of one of the haemagglutinating strains suggested that the strain is composed of haemagglutinating (H^+) and non-haemagglutinating (H^-) particles. This was confirmed by further experiments aimed at the separation of H^+ and H^- particles by (i) adsorption to, and elution from, the Ca-phosphate column; (ii) by adsorption to erythrocytes; and (iii) by the limiting dilution method. The H^+ particles were eluable from the Ca-phosphate column at 0.2 M concentration whereas the bulk of the H^- particles required for elution a concentration of about 0.5 M. The H^+ particles were adsorbable to human erythrocytes whereas the H^- particles were practically not adsorbable. H^+ and H^- lines could be produced by passages with the limiting dilutions. The two lines thus obtained proved to be homogeneous when carried over 14 (H^+) and 9 (H^-) consecutive passages, respectively. In monkey kidney cell cultures the H^- particles were reproduced faster, attained higher titres, and produced complete cell destruction, in contrast to the H^+ particles. The H^- particles were, the H^+ particles were not, inhibited by the agar polysaccharide. Some antigenic difference was also demonstrable.

It is concluded that all variation described for echovirus type 6 may be attributed to the existence of H^+ and H^- particles; the occurrence of two kinds of particle might be a general phenomenon in the enterovirus family.

Recently, intratypic divergences have been observed within several echovirus types. In this respect type 6 has been studied most thoroughly. First the antigenic difference (prime strains) was described [1, 2] and studied in detail [3]. In the course of these studies the "antigenic phase" character (S and B phases) was found to be closely associated to other characters, *viz.* the velocity and intensity of reproduction, the completeness of the cytopathic (CP) effect, the sensitivity to the inhibitory effect of the agar polysaccharide, *etc.* [3, 4, 5, 6]. Besides, strain differences in the haemagglutinating (HA) capacity were demonstrated within type 6. Although GOLDFIELD *et al.* [7], who were the first to report on the agglutination of human erythrocytes by echoviruses, found all the type 6 strains tested to agglutinate, LAHELLE [8, 9] and, later, others [4, 10, 11, 12, 30] could not reproduce this phenomenon with every strain of type 6. The relationship between HA capacity and other strain characteristics is not yet clear.

The present studies have shown that from suspensions of echovirus type 6 strains two kinds of virus particle, *viz.* haemagglutinating (H^+) and non-haemagglutinating (H^-) particles, are separable. The H^+ and H^- particles differ from each other in other characteristics, too, such as antigenic structure, reproducing capacity, sensitivity to the agar inhibitor, *etc.*

Materials and methods

Viruses. The D'Amori strain, the prototype of echovirus type 6, was kindly supplied by M. P. CHUMAKOV (Moscow). The strain had undergone 40 tissue culture passages altogether. Ten further strains of echovirus type 6 were isolated in this laboratory in monkey-kidney primary cell cultures, in the course of screening of healthy subjects. The strains were identified by the neutralization test. The methods were published in detail elsewhere [13].

Tissue cultures. Primary cultures of rhesus kidney cells were used throughout. As maintenance medium Parker's 199 was used, except when the inhibition by agar extract was examined.

Virus titration. Three, in certain cases five, tube cultures were inoculated with each dilutions of tenfold series. The results were registered after microscopic examination on the 10th day. TCID₅₀ was calculated according to the REED-MUENCH formula [14].

The haemagglutination (HA) test was carried out by means of TAKÁTSY's Microtiterator [15]. Human blood was taken with Na citrate, the erythrocytes were washed three times and were diluted to result in a 1 per cent suspension. As diluent, veronal buffer of pH 7.2, containing also Ca⁺⁺ and Mg⁺⁺ was used. For HA titrations twofold dilution series were made and the results were read after an incubation with the erythrocytes at 37° C for 1 hour.

Chromatographic analysis was made in the Ca-phosphate column as described earlier [16]. As eluent, a series of pH 7.2 phosphate buffer solution with gradually rising molar concentration (0.001, 0.1–0.7 M) was used.

Agar extract for inhibition experiments was prepared as recommended by SCHULZE and SCHLESINGER [17].

Immune sera. Rabbit and mouse immune sera were used. The schedule of immunization has been published [13].

Results

Studies on the correlation between the HA capacities and infective titres of echovirus type-6 strains. Out of the 10 type-6 strains isolated by us only three were found to possess HA activity. Neither the rest nor the prototype (D'Amori) agglutinated human red cells. These observations conform to earlier data [4, 10, 11, 12, 30].

Table I
Infective and HA titres of echovirus type-6 strains

Strain	Passage No.	log TCID ₅₀ /0.1ml	HA titre*
D'Amori	40	6.7	—
33/61	4	6.0	—
676/61	3	5.5	16
1167/61	5	6.2	—
1232/61	3	4.5	—
1463/61	3	6.5	—
2060/61	4	4.7	—
3776/61	3	5.2	—
3887/61	2	4.2	64
3947/61	2	4.2	64
3961/61	4	6.5	—

* Reciprocals, — = < (1 : 2)

Table I shows no correlation between infectivity and HA capacity for the type-6 strains tested, e.g. strains 3887/61 and 3947/61 agglutinated erythrocytes in spite of their low infectivity while the prototype as well as strains 1463/61 and 3961/61 failed to haemagglutinate although their infective titres were more than 100 times higher.

BUSSEL *et al.* [4] and GAUDIN *et al.* [12] have shown that the degree of haemagglutinin production may change during consecutive passages; to retain HA capacity, diluted inocula should be used. The fact that our own strains had undergone 3 to 5 passages in monkey-kidney cell cultures and for these passages undiluted inocula were used raised the possibility that the non-haemagglutinating strains might have lost their HA capacity in the course of the passages.

To elucidate this question, one of the haemagglutinating strains, 3887/61, was carried over further passages using undiluted inocula (Table II). In the

Table II

Infective and HA titres in the course of the passages of strain 3887/61 of echovirus type-6

	Passage			
	2	3	4	5
—log CPID ₅₀ /0.1 ml	4.2	5.2	6.0	6.5
—log HA titre	1.8	1.8	1.5	1.5
log I/H	2.4	3.4	4.5	5.0

course of four passages the infective titres increased up to 100 fold while the HA titres remained practically unchanged. Consequently, the ratio infective titre per HA titre (I/H) rose from $10^{2.4}$ to $10^{5.0}$.

This finding, though it confirmed that passages with undiluted inocula were unfavourable for the HA capacity, has rendered improbable the supposition that our non-haemagglutinating strains had lost their HA activity as a result of 3 to 5 similar passages.

In our opinion the significant rise of the I/H ratio can be explained in two ways if we assume that the virus particle itself is the haemagglutinating factor [7, 9].

(a) Every particle takes part in the HA but, because of some changes in surface structure, the number of particles required for visible HA rose from approximately 100 to 100,000 during the passages.

(b) Only certain particles possess HA capacity, and passages carried out with undiluted inocula are unfavourable for the haemagglutinating particles.

We wished to obtain further information by observing the development of both the infective and HA titres in the cells and in the medium. For this

purpose 30 tube cultures of monkey-kidney cells were inoculated each with 0.1 ml of the undiluted material of strain 3887/61 (designated as passage 3 in Table II); another 30 tube cultures were inoculated with the same material but diluted 1 : 100. After incubation at 37°C for four hours the cultures were washed three times with Hanks' solution; then Parker's 199 was added. Three tubes were removed at the periods shown in Fig. 1. The medium of the three

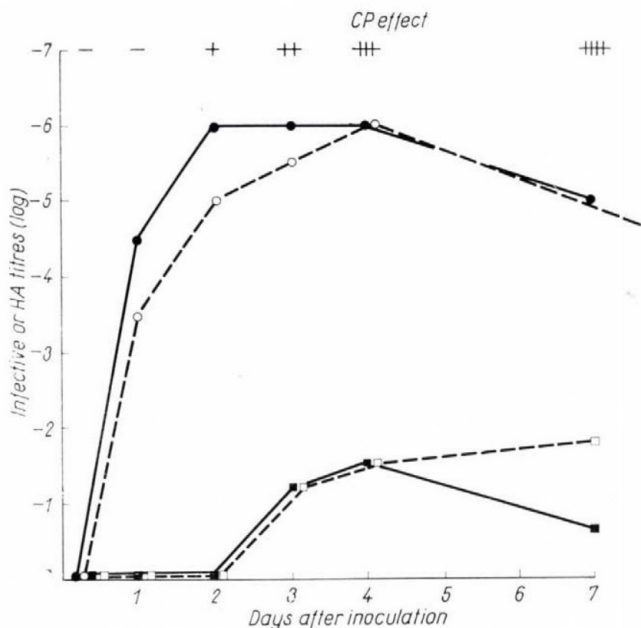


Fig. 1. Development of infectivity and HA activity in the cells and in the medium (strain 3887/61, multiplicity ratio, 0.01)

- — ● cell-fixed virus
- — ○ released virus
- — ■ haemagglutinin in the cells
- — □ released haemagglutinin

tubes was pooled, the cells were washed once, and a volume of Parker's 199 equal to the volume of the original medium was added. The cell suspensions thus obtained were frozen and thawed three times, and centrifuged. The experiments performed with 1 : 100-diluted inocula (multiplicity ratio, 0.01) are shown in Fig. 1.

The results agreed with those obtained by other authors for echovirus type-6 [4, 9] and for other echovirus types [11, 18]. The appearance of infectivity preceded that of the HA; the former attained appreciable titres before the first CP changes had become visible. The HA titre began to rise thereafter. To explain the late appearance of the HA capacity, it might be supposed that the initial number of infectious particles was not sufficient to produce HA.

However, an analysis of the data of Fig. 1 renders this assumption unlikely. From the third day of observation on the I/H ratio fluctuated between $10^{4.4}$ and $10^{4.8}$ for the cells and between $10^{3.2}$ and $10^{4.3}$ for the medium. Infective titres obviously exceeding these values were shown by both the medium (10^5) and the cells (10^6) as soon as on the second day, without any sign of HA.

This observation is hardly compatible with explanation (a) but conforms to explanation (b). The rise of the I/H ratio in the course of consecutive passages is also well explainable by the supposed existence of two kinds of particle. This view is supported also by the following experiments. The non-haemagglutinating suspensions harvested on the first day after inoculation were inoculated into tissue cultures. The resulting virus suspensions showed no HA in contrast with those obtained from cultures that had been inoculated with the haemagglutinating suspension harvested on the 7th day after inoculation.

The above results encouraged our attempt to separate the supposed haemagglutinating (H^+) and non-haemagglutinating (H^-) particles from each other.

Attempts to separate H^+ and H^- particles

Chromatographic experiments. In these experiments the second passage material (Table II) of the 3887/61 strain was used. The TCID₅₀ values and the HA titres were determined for each of the eluted fractions (Fig. 2).

The infectivity curve was biphasic. The peak of the first wave was at 0.2 M, that of the second wave at 0.5 M, phosphate buffer. HA activity was demonstrated in the fractions corresponding to the first wave, and the highest

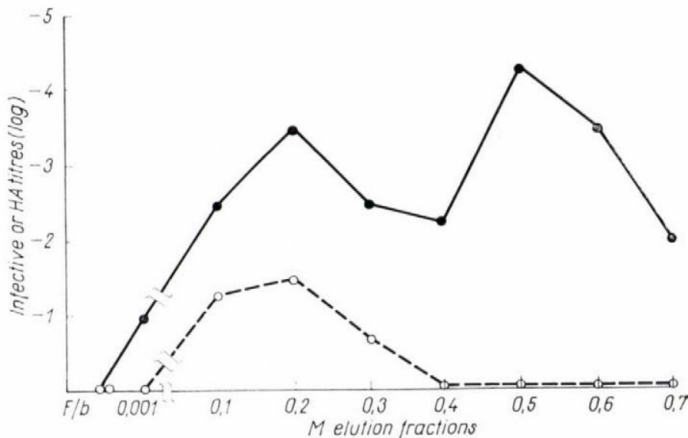


Fig. 2. Infectivity and HA titres of the fractions of virus eluted from the Ca-phosphate column with phosphate buffers of various molarities (strain 3887/61)

●—● infectivity
○—○ HA

titre coincided with the peak of the wave. The I/H ratios were $10^{1.3}$, $10^{2.0}$, and $10^{1.8}$ in fractions 0.1 M, 0.2 M, and 0.3 M, respectively. In the fractions corresponding to the second wave no HA activity could be detected. It can therefore be concluded that the 3887/61 strain contains two kinds of virus particle, the H^+ particles being eluable with buffers of relative low molarity while elution of the H^- particles requires a higher molarity.

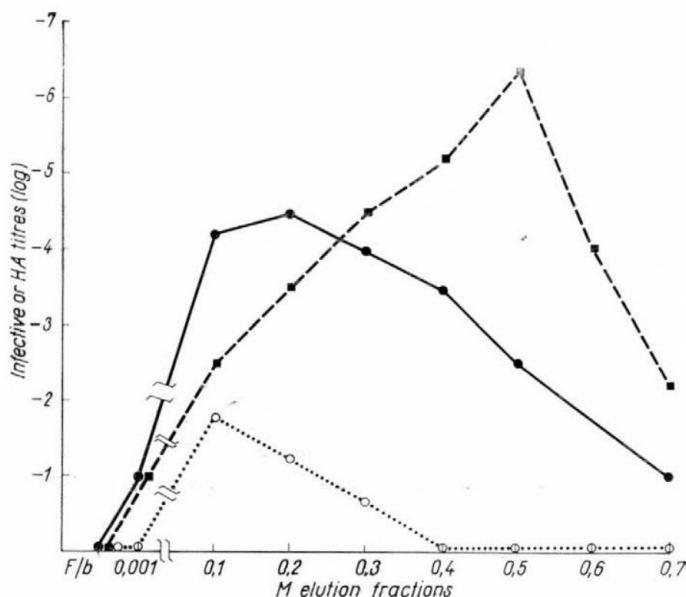


Fig. 3. Elution from the Ca-phosphate column of haemagglutinating (3947/61) and non-haemagglutinating (3961/61) strains

- infectivity, haemagglutinating strain 3947/61
- HA capacity, haemagglutinating strain 3947/61
- infectivity, non-haemagglutinating strain 3961/61

This experiment was repeated with another haemagglutinating strain (3947/61) and with a non-haemagglutinating strain (3961/61). The results are shown in Fig. 3.

Out of the fractions of the 3947/61 strain those eluted with low-molarity buffer showed the highest titres and HA. In contrast to this, the bulk of the infective virus of strain 3961/61 was recovered in the high-molarity fractions and these fractions gave no HA. The lack of a second wave in the curve of strain 3947/61 might indicate that this strain contains fewer H^- particles than strain 3887/61 does. However, a considerable amount of infectious virus was eluted in the low-molarity fractions also from the 3961/61 strain, and these fractions failed to agglutinate erythrocytes. This observation suggests that the selection of the H^+ and H^- particles by the Ca-phosphate column was not complete. It should be noted that the summation of the curves of strains

3947/61 and 3961/61 (Fig. 3) results in a curve very similar in shape to the curve of the 3887/61 strain (Fig. 2).

Adsorption to human erythrocytes. One ml of undiluted, washed erythrocytes was added to 1 ml undiluted virus suspension prepared from strain 3887/61. After one hour at 37°C the erythrocytes were sedimented by centrifugation (1500 r. p. m. for 10 minutes). The supernatant was removed and the erythrocytes were washed three times with saline of +4°C. Both the supernatant and the sediment were titrated for infectivity and the supernatant also for HA. The starting material had an infective titre of $10^{-4.2}$ and a HA titre of 1:64. The infective titre of the supernatant was 10^{-3} , but this was not associated with HA activity; 0.1 ml of the undiluted erythrocytes contained $10^{5.5}$ TCID₅₀. (The increase in the total number of infectious units should be attributed to the error of the titrations.) In a similar experiment with the non-haemagglutinating strain 3961/61 the infectivity of the virus suspension was not altered by treatment with erythrocytes and 0.1 ml of the erythrocyte sediment contained as little as 10 TCID₅₀ of virus. It can be concluded that adsorption to human erythrocytes also results in separation of H⁺ and H⁻ particles. The latter are hardly adsorbed to the erythrocytes.

Separation by passages of limiting dilution. Monkey-kidney cultures were infected with approximately 1 TCID₅₀ of the strain 3887/61 each. Of the resulting cultures those showing both infectivity and HA and those showing only infectivity, were separately pooled and the pools were titrated for infectivity. The cultures which proved to have been infected with the limiting dilution were subjected to the same procedure. Thus, two passages were performed with limiting dilutions. Out of the resulting virus suspensions the H⁺ suspension contained $10^{6.3}$ TCID₅₀ per 0.1 ml and its HA titre was 1:128, whereas 0.1 ml of the H⁻ line contained 10^7 TCID₅₀, but gave no HA.

*Examination for homogeneity of the H⁺ and H⁻ fractions
obtained by different methods*

A virus suspension prepared from the 3887/61 strain was adsorbed to the Ca-phosphate column. The fraction that was eluted by the 0.2 M buffer and that eluted by the 0.6 M buffer was separately inoculated into monkey-kidney cell cultures and consecutive passages were carried out with (a) undiluted inocula and (b) 1:100-diluted inocula. The dynamics of infectivity and HA titre were followed through 5 passages (Table III).

The infectivity of the 0.2 M fraction showed a consistently increasing tendency while the HA titre tended downwards. The non-haemagglutinating 0.6 M fraction gave rise to non-haemagglutinating virus up to the 4th passage. At the 5th passage, however, some HA was demonstrable. Infectivity increased in this case, too.

Table III

Infective and HA titres in the course of consecutive passages started with 0.2 M and 0.6 M fractions of strain 3887/61

Fraction eluted at molarity		Passage				
		1	2	3	4	5
0.2 M	—log TCID ₅₀ /0.1 ml	3.5	4.0	5.0	5.5	5.5
	—log HA titre	1.5	1.8	1.2	0.6	0.25
0.6 M	—log TCID ₅₀ /0.1 ml	3.5	5.0	5.0	5.5	5.5
	—log HA titre	0	0	0	0	0.25

In the course of the passages with diluted inocula the HA titre remained practically unchanged up to the 5th passage in the line initiated with the 0.2 M fraction while in the line of the 0.6 M fraction HA appeared as soon as in the 2nd passage and the HA titre rose consistently thereafter. The infective titres showed the same tendency as in the course of passages performed with undiluted inocula. These results have confirmed our earlier supposition that the fractions eluted from the Ca-phosphate column were inhomogeneous.

The virus suspension (strain 3887/61) unadsorbed by erythrocytes also proved inhomogeneous. Though the starting material possessed no HA activity, haemagglutinating suspensions were produced during consecutive passages.

The H⁻ line obtained from the 3887/61 strain by passages with limiting dilutions produced no haemagglutinating suspensions up to the 8th passage. In the 9th passage, however, traces of haemagglutinin were detectable. The H⁺ line, on the other hand, retained its character through all the 14 passages. In the course of these passages neither the infectivity nor the HA titre showed any significant fluctuation. The I/H ratio ranged from 10^{3.8} to 10^{4.0}.

On these findings the H⁺ and H⁻ lines that had been obtained by the limiting dilution method were accepted as practically homogeneous, thus suitable to study the characteristics of the H⁺ and H⁻ particles.

*Some characteristics of the H⁺ and H⁻ particles separated
from the 3887/61 strain*

The following experiments were performed with the first passage materials of the H⁺ and H⁻ lines obtained by the limiting dilution procedure.

The morphology of the CP changes in the monkey-kidney cell culture was independent of the H⁺ or H⁻ character of the inoculum. However, the cell destruction due to H⁺ particles developed slower and never involved every cell.

Some difference was found between H^+ and H^- particles in the rate of adsorption to monkey-kidney cells. To study this phenomenon, tube cultures were washed with Hanks' solution and infected partly with H^+ , partly with H^- suspension. The inoculum uniformly contained 10,000 TCID₅₀ in a volume of 0.2 ml. The cultures were incubated at 37°C and four tubes were removed at intervals. These were carefully rocked in hand to wash of the unfixed particles. Then the medium of the four cultures was pooled and titrated. The one-

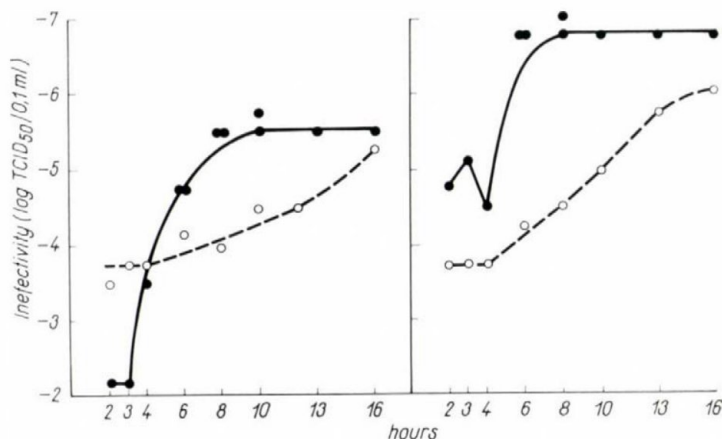


Fig. 4. Reproduction of the H^+ and H^- virus particles in monkey-kidney cell cultures

●—● cell-fixed virus
○—○ released virus

hour values were repeatedly determined using 10 tube cultures for each of the virus dilutions. According to these experiments the H^+ particles were adsorbed somewhat slower than the H^- particles. By the end of the first hour 75 per cent of the H^- particles and only 45 per cent of the H^+ particles had been adsorbed.

The course of reproduction was also followed. Monkey-kidney tubes were washed with PBS and inoculated with 0.1 ml virus suspension each. The multiplicity ratios were 52 TCID₅₀ per cell and 28 TCID₅₀ per cell for the H^- and H^+ lines, respectively. After one hour of adsorption the unadsorbed inoculum was removed, the monolayer was washed three times with PBS, and 1 ml Parker's 199 was added. The cultures were then re-incubated at 37°C and three of the tubes were removed at intervals. The medium of these cultures was removed, pooled, centrifuged at 1500 r. p. m. for five minutes to remove cell debris, and stored at -20°C until used for titration. The cell monolayer was washed twice, then 1 ml Parker's 199 was placed in each tube and the tubes were stored at -20°C until used. On the day of the experiment the cell fraction was frozen and thawed three times and centrifuged. The supernatants

of the three tubes were pooled. In the following the preparations thus obtained were signed "cell-fixed virus".

Fig. 4 shows the course of reproduction for the H^+ and H^- particles. Latency lasted for $3\frac{1}{2}$ to 4 hours, irrespective of line. However, the H^- particles multiplied faster, and attained higher titres, than the H^+ particles. According to this experiment the duplication time of cell-fixed virus amounted to 16 minutes for H^- particles and to 36 minutes for H^+ particles, whereas the corresponding values for released virus were 87 and 141 minutes, respectively.

To reveal possible antigenic differences, cross neutralization tests were carried out. The anti- H^+ serum was prepared by inoculating mice with H^+ suspensions whereas the immune serum prepared in rabbits with the prototype (D'Amori) strain served as anti- H^- serum. The sera were tested against 100 TCID₅₀ of virus, uniformly. The results are shown in Table IV.

Table IV

Cross neutralization tests with the H^+ and H^- variants separated from strain 3887/61 of echovirus type 6

Immune serum	Neutralization titres* of sera against	
	H^+	H^-
	virus	
anti- H^+ mouse	640	320
"D'Amori" rabbit	1000	32000

* Reciprocals

Only the anti- H^- serum made distinction between H^+ and H^- particles. Here the ratio homologous titre per heterologous titre was 32/1. The antigenic difference is thus obvious although the H^+ serum made no appreciable distinction between the two lines ("one-way-cross phenomenon"). These observations are analogous to those published by KARZON *et al.* [3].

The effect of the agar inhibitor on the reproduction of the H^+ and H^- lines was also studied. Both variants were titrated simultaneously, both with, and without, agar extract. The medium of the control cultures contained 0.11 per cent NaHCO_3 and 2 per cent calf serum in Earle's solution; the medium of the other series was the same, except that 10 per cent aqueous agar extract was added. The cultures were incubated at 37° C for eight days. As Table V shows, the agar extract did not influence the reproduction of the H^+ variant while obviously inhibited the H^- line. In another experiment, when the medium contained 20 per cent agar extract, inhibition was more pronounced.

The foregoing experiments were performed in stationary cultures. When roller tube cultures were used, the agar polysaccharide caused no observable inhibition in the reproduction of the H^- variant.

Table V

Inhibition by agar extract of viral reproduction in monkey-kidney stationary cell cultures

Virus	Agar extract vol. per cent	CP changes in cultures inoculated with						
		-1	-2	-3	-4	-5	-6	-7
		dilutions of virus*						
H ⁺	—	++++	++++	++++	++++	++++	++++	—
	10	++++	++++	++++	++++	++++	++++	—
H ⁻	—	++++	++++	++++	++++	++++	++++	—
	10	++++	+++	++	±	—	—	—

* Dilution in log₁₀; read on the 8th day after inoculation

In Table VI the characteristics of the H⁺ and H⁻ particles are summarized comparatively.

Table VI

Characteristics of H⁺ and H⁻ particles of echovirus type 6

Phenomenon	H ⁺	H ⁻
HA	positive	negative
CP effect	incomplete	complete
Adsorption to cells	slow	rapid
Reproduction	slow	rapid
Infective titre	low	high
Sensitivity to agar inhibitor	resistant	sensitive
Sensitivity to antibodies	less sensitive	sensitive
Elution from Ca ₃ (PO ₄) ₂ column	mainly in 0.2 M fraction	mainly in 0.5 M fraction

Discussion

The existence of two kinds of echovirus type-6 particles makes it possible to give universal explanation for some, often contradictory, phenomena described by other investigators.

Since the discovery of the echovirus-HA by GOLDFIELD *et al.* [7] several authors [4, 8, 9, 10, 11, 12, 30] have pointed out that only some of the echovirus type-6 strains possess this capacity. These observations can be understood if it is supposed that strains may purely be composed of H⁺ or H⁻ particles

or may contain both particles at variable ratio. If so, the concentration of the H^+ particles in a virus suspension should determine whether or not the suspension agglutinates human erythrocytes. In strains consisting of mixed particles the I/H ratio depends on the percentage of H^+ particles in the mixture. Consequently, the strains containing practically no H^+ particles (e.g. the D'Amori strain) do not agglutinate erythrocytes even at levels of 10^8 to 10^{10} TCID₅₀ per ml [9]. For the H^+ line established by passages with limiting dilutions, on the other hand, the I/H ratio was approximately 10^4 and this value was consistent over 14 passages. For this reason this line may be regarded as a pure H^+ line, and 10^4 infectious units per 0.1 ml may be accepted as the lowest concentration of H^+ particles causing visible HA. Accordingly, in virus suspensions containing both H^+ and H^- particles the I/H ratio should be higher than 10^4 .

The divergent reproductive capacities of the H^+ and H^- particles in monkey-kidney cell cultures may explain that in the course of the passages started with the original suspension as well as the 0.2 *M* fraction of strain 3887/61 the HA titre was consistent or even tended downward while infectivity was increasing. It can also be understood why the HA appeared later than the infectivity had begun to rise in the case of our strain (3887/61) and of those studied by BUSSEL *et al.* [4] and LAHELLE [9].

The faster reproduction and higher titres of H^- particles may explain why some originally haemagglutinating strains were losing that capacity during consecutive passages [12] and why the use of diluted inocula was favourable for haemagglutinin production [4, 11].

The H^+/H^- ratio of a resulting culture may depend on the conditions of cultivation (tissue culture [18, 19, 20], size of inoculum *etc.*); e.g. instable conditions may explain why the prototype strain of echovirus 6⁷ (DiMeo) was found to haemagglutinate in one laboratory, but not in another one [11].

KARZON *et al.* [3], while studying the antigenic variation of several strains of echovirus type 6, found that the strains could be classified into two groups on the basis of their sensitivity to homotypic antibodies. They called the strains well-neutralizable only by the homologous (strain-specific) antibodies S- (specific) phase strains whereas those also neutralizable with antibodies produced against other homotypic strains B- (broad) phase strains. The same authors demonstrated that the phase character is associated with other biological characters, *viz.* S-phase strains grow slower in monkey-kidney cultures, and attain lower titres than B-phase strains and produce an incomplete CP effect. However, the phase character proved to be instable; S-phase strains turned into B-phase after 6 to 10 passages. The same investigators reported later that under the agar overlay S-phase strains produced large plaques (m^+ marker) in contrast with the small plaques (m marker) produced by B-phase strains. The m marker was attributed to the agar polysaccharide

[5]. SUTO *et al.* [6] reported that m^+ strains turned into m strains in the course of passages.

Comparison of the biological and serological properties of S-phase strains with those of our H^+ line has shown a complete agreement, and the S—B transformation may be regarded as analogous to the overgrowth of H^+ particles by H^- ones. It seems therefore reasonable to attribute the S—B variation, too, to the existence of two kinds of particles in the same viral population. In addition, the B-phase strains of the KARZON group [4] either gave no HA or had extremely high I/H ratios (10^6 — 10^7) whereas their S-phase strains, except one, agglutinated erythrocytes and had low (approximately 10^4) I/H ratios. The single nonhaemagglutinating S-phase strain was a derivative of the DiMeo strain, which, as mentioned, was found to haemagglutinate in another laboratory [11].

Intratypic existence of different particles is not a unique observation in the Enterovirus family. CHOPPIN and EGGERS [21] separated different particles from coxsackievirus type B-4 whereas JOHNSON and LANG [20] from coxsackievirus type A-21 (Coe virus); in the former case the relationship to HA was not studied.

The facts that (i) besides echovirus type 6 other enterovirus types have been found to include haemagglutinating and non-haemagglutinating strains; that (ii) fluctuation in the HA activity during consecutive passages has been observed in other types, too, [10, 11, 12, 19, 22, 23, 25]; and (iii) that ever more types formerly thought to be non-haemagglutinating have been proved to include haemagglutinating strains [12, 23, 24, 26] suggest the existence of two kinds of particle in most of the enterovirus types. We have even experimental evidence of the existence of different particles within the echovirus types 3, 7, 11, and 12; the concerning data will be published in detail.

The question of the genetic stability of H^+ and H^- particles needs further study. The mechanisms of intracellular reproduction are obviously different for H^+ and H^- particles as suggested by the different velocities of reproduction and by the observation that only H^- particles are inhibited by the agar polysaccharide, which also makes distinction between variants of polioviruses (d variants), EMC viruses [28], and coxsackievirus type B-4 [21]. The agar polysaccharide is a sulphated polysaccharide [17, 27], and this, like other polyanions, has been found to inhibit intracellular enzymes. The inhibition of the virus reproduction might be due to such an enzyme inhibition. The inhibition due to the agar polysaccharide could be suspended by dextran [17, 21, 29] or protamine [5]. Our finding, that the inhibition of H^- particles is not demonstrable in roller tube cultures, suggests that an increase in the oxydative metabolism of the host cell might also suspend the inhibition.

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GROUP AND TYPE DISTRIBUTION OF HAEMOLYTIC STREPTOCOCCI IN HUNGARY DURING THE YEARS 1958—1963

By

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Summary. The serological typing of 1377 haemolytic streptococcal strains isolated during the years 1958—1963 has been carried out. Of the examined strains 82.4 per cent belonged to group A. Within group A, by M antigen determination 48.7 per cent, by T antigen determination 84.5 per cent, of the strains were typed; 7.5 per cent of the cultures were untypable by both methods. As to the incidence of the predominating types (complexes 5, 11..., 3, 13, B₃₂₆₄, 8, 25, Imp. 19 and types 3, 12 and 1), our results agreed closely with those reported for 1960—1961 by RÖTTA in Czechoslovakia. No significant differences were noted as regards the distribution of the predominating types in different areas of Hungary. The observations are considered to have furnished a basis for an improved laboratory study of streptococcal diseases and their clinical and epidemiological problems in Hungary.

In view of their frequency and dangerous sequelae, streptococcal infections comprise a serious health problem all over the world. The significance of these infections is best estimated by surveying the incidence of scarlet fever, which has been recorded in Hungary ever since 1881. (Of streptococcal infections and their complications, scarlet fever, puerperal fever and rheumatic fever are compulsorily reported in Hungary.) The mortality of scarlet fever in the years between the end of the last century and 1920 was 40—80 per 100,000 inhabitants. In 1923—24 a striking change occurred, as this value gradually decreased to 1—3.

In 1962 the mortality of scarlet fever was nil. The scarlet fever epidemics occurring in 1948—1951 did not alter considerably these death rates. The morbidity of the disease, however, is still high. In 1962 the number of reported cases was 21,000. Streptococcal infections of other kinds are also of great importance if the unrecorded but presumably high number of cases of streptococcal sore throat, erysipelas, nephritis, etc. are considered.

In these days research work is mainly focussed upon staphylococcal infections although, in our opinion, the fight against streptococcal diseases has not lost of its importance. As explained by WILLIAMS *et al.* [2] and ourselves [3], this is due to the decreasing virulence of streptococci in the last 3—4 decades and, on the other hand, that penicillin is still very effective against streptococcal infections.

In Hungary, MIHÁLYFI and FÜRÉSZ [4] were the first to study the group and type distribution of haemolytic streptococci by use of sera obtained from the Institute of Microbiology and Epidemiology, Prague. They performed

the determination of T antigens of *Str. pyogenes* strains isolated during the 1949–1951 scarlet fever outbreaks and in 1953. In view of the high morbidity of streptococcal infections in this country, it seemed desirable to perform more detailed investigations into the serological distribution of these organisms.

Materials and methods

Preparation of C, T and M antisera was commenced in 1960. The necessary type cultures were obtained from different institutes. In addition to checking in our laboratory, the sera were sent for a comparative control to the Institute of Epidemiology and Microbiology, Prague.

Cultures. In order to follow the changes in the distribution of serotypes for a longer period, the collection of strains was begun in 1958. Most of these cultures originated from patients admitted to the László Hospital for Infectious Diseases, Budapest. These strains were lyophilized and stored until 1961, when systematic group and type determination was begun. In 1961 the Regional Public Health Laboratories were asked to send their freshly isolated streptococcal strains to this laboratory together with some records of the origin of the cultures. The strains were received on blood agar slants or Loeffler media. Further strains were isolated from our own routine material. Immediately after arrival or isolation, the cultures were streaked on to ox blood agar plates, then grouping and typing were performed. The examined strains were isolated in the months December and May.

Group identification. *Str. pyogenes* (group A) strains were distinguished from other strains by examining their bacitracin sensitivity. When the sensitivity to bacitracin was doubtful or the strain was resistant to this antibiotic, the precipitation reaction with LANCEFIELD or FULLER extracts was performed in group sera A–S. For a comparison, the MAXTED extracts [5] of group A, C and G strains isolated in 1958 were also tested by precipitation. With strains belonging to type 2 or complexes 4.24... or 8.25, Imp. 19, the result of grouping was checked by performing the test with FULLER extracts.

Type identification. T antigens were determined by GRIFFITH's agglutination technique [6]. The slide agglutination was performed with homogeneous, trypsinized streptococci cultured in 5 ml TODD–HEWITT broth for 24–48 hours at 30° C. The suspension was tested first with 6 pool sera (T, U, W, X, Y, Z), then with the monovalent sera of the pool which caused agglutination.

The M antigen of all *Str. pyogenes* strains was determined with LANCEFIELD's precipitation technique [7]. The reaction was carried out with the hydrochloric acid extract of the deposit from 40 ml TODD–HEWITT broth culture that had been incubated for 16 hours at 37° C. The extract was precipitated with anti-M sera in SWIFT capillaries [8]. After an incubation for two hours at 37° C, the tubes were left in the refrigerator overnight and then were final readings made. The precipitation reaction was carried out, in addition to some control sera, with type sera corresponding to the result of agglutination. If a strain could not be determined by agglutination, it was tested for precipitation with all sera. If the strain gave a specific precipitation with one of the M sera, or agglutination with one of those T sera which have type-specific character, it was regarded as belonging to a separate type. If the strain was untypable by the M antigen, and by agglutination it was shown to possess a complex T antigen, it was included in the corresponding complex. The strain was regarded as untypable when neither its M nor its T antigens were definitely recognized. It should be noted that despite their definite precipitation reaction, type 4 strains were included in complex 4.24... because our anti-M serum 4 contained more likely anti-T than anti-M factors.

Results

During the examination period 1377 haemolytic streptococcal strains were collected. Table I shows that 82.4 per cent of the strains belonged to group A. Group C and G strains were almost equal in frequency (5.8 and 5.9 per cent). The relatively high incidence of group B strains (5.1 per cent) was noteworthy. Altogether 10 strains (0.8 per cent) belonged to other, namely to D, H, and K groups.

Table I
Group distribution of haemolytic streptococcal strains

Group	Number	Per cent
A	1135	82.4
B	70	5.1
C	80	5.8
G	82	5.9
Other	10	0.8
Total	1377	100.0

Table II presents the type distribution of the 1135 A group strains. The types are grouped according to the technique of type determination (precipitation, agglutination or both methods combined). By M and T antigen identification 92.5 per cent of *Str. pyogenes* strains was typed. The incidence of untypable strains was 7.5 per cent. If each complex is regarded as one type, the 1135 strains were distributed among 28 different types. Types 24, 33, 39 and 47 were determined only by precipitation. On the basis of agglutination, types 2, 9 and 22 were defined as separate serotypes. During the observation period complexes 5, 11, 12 . . . , 3, 13, B₃₂₆₄ and 8, 25, Imp. 19 and types 1, 3 and 12 predominated. Serotypes occurring with a frequency of more than 5 per cent were considered prevalent types. The yearly distribution of these types is shown in Table III.

As in May and December the percentage distribution of prevalent types collected in 1958 or 1962 was similar, summarized results are presented. From Table III it may be seen that the complex 5, 11, 12 . . . , as compared to its incidence in 1958, became more frequent in the following years. In contrast, the incidence of type 1 showed an opposite tendency. With type 12 and complex 3,13, B₃₂₆₄ the maximum frequency was noted in 1961 and 1963. Type 3 (including type 3R) and complex 8, 25, Imp. 19 were evenly distributed during the examination period. It should be noted that the incidence in the various years of the 6 predominating types presented in Table III was 55–62 per cent of the total number of A group strains.

In the spring of 1963, the so far infrequent types 28 and 29 emerged with an incidence of 10.0 and 8.2 per cent, respectively.

The distribution of the prevalent types in various areas of Hungary is shown in Fig. 1. The data refer to the years 1961–1963, as the overwhelming majority of strains isolated in 1958 had originated from patients treated in Budapest.

Table II
Type distribution of Str. pyogenes strains
(1958—1963)

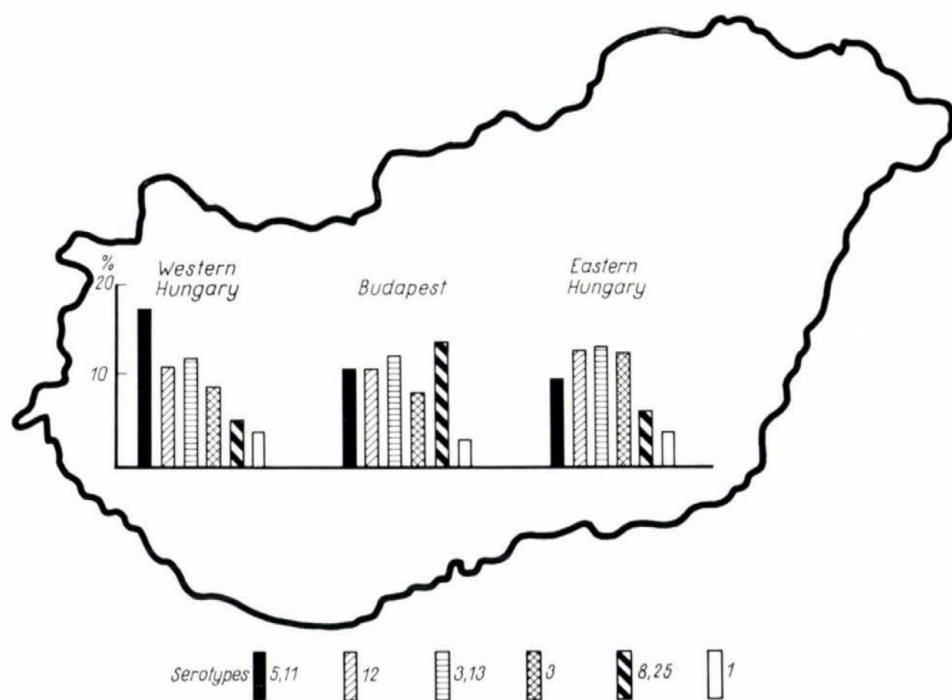
Type	Number of strains determined by antigens			Total	
	M	T	M and T	No.	%
1		8	78	86	7.6
2		21		21	1.9
3M	5		86	91	8.0
3R			8	8	0.7
3, 13, B ₃₂₆₄		122		122	10.7
4, 24, . . .		30	9	39	3.4
5	1		4	5	0.4
5, 11, . . .		132		132	11.6
6	11	1	45	57	5.0
8, 25, Imp. 19		96		96	8.5
9		6		6	0.5
11	1		1	2	0.2
12	2		125	127	11.2
13	1		1	2	0.2
14	1	14	23	38	3.3
15	1		1	2	0.2
15, 17, . . .		40		40	3.5
18	1	18		19	1.7
19	50		7	57	5.0
22		9		9	0.8
24	2			2	0.2
26			3	3	0.3
28	1		27	28	2.5
29	16		21	37	3.3
33	2			2	0.2
39	1			1	0.1
44			5	5	0.4
46			12	12	1.0
47	1			1	0.1
Total typable	97	497	456	1050	92.5
Untypable				85	7.5
Total				1135	100.0

Table III

Yearly percentage distribution of *Str. pyogenes* strains prevalent in Hungary

Type	1958 (259)*	1961 (289)*	1962 (428)*	1963 (159)*	Total (1135)*
5, 11, 12, 27, 44	9.3	5.2	15.7	16.4	11.6
12	9.7	18.7	9.6	4.4	11.2
3, 13, B ₃₂₆₄	4.7	10.7	12.6	15.7	10.7
3	5.4	6.9	11.2	10.7	8.7
8, 25, Imp 19	8.1	10.0	9.6	3.1	8.5
1	21.1	3.9	3.0	4.4	7.6
Other types	41.7	44.6	38.3	45.3	41.7
Total	100.0	100.0	100.0	100.0	100.0

* Total number of A group strains examined in the corresponding year.

Fig. 1. Distribution of prevalent *Str. pyogenes* types in different areas of Hungary in 1961-1963

The number of strains did not suffice for evaluating the results according to counties. The data obtained in Budapest were therefore compared with the summarized data of the eastern and western parts of the country.

Fig. 1 clearly shows that complex 5, 11, 12, ... predominated in West Hungary. Complex 8, 25, Imp. 19 was most frequently encountered in Budapest. Other prevalent types were more or less evenly distributed.

Discussion

The purpose of the introduction of group and type determination of haemolytic streptococci in our laboratory was to establish, (i) whether results comparable with those published in the literature could be achieved by the use of our method and our sera; (ii) whether changes have occurred in the distribution of *Str. pyogenes* since the 1953 studies of MIHÁLYFI and FÜRÉSZI [4]; (iii) the occurrence of types prevailing in Hungary and the neighbouring countries. (iv) Finally, we aimed at providing our epidemiologists with reliable laboratory data for eventual streptococcal epidemics.

Of the 1377 strains, 62.4 per cent belonged to group A. In the various years and seasons this value was between 74.6 and 90.4 per cent. This frequency is similar to that found by other authors [9-14]. The incidence of human group C and G strains was 5.8 and 5.9 per cent, respectively. ROTTA's result was higher (14 and 7.9 per cent) for the incidence of these groups, and lower (2.5 per cent) for group B. Our material included strains isolated from patients and carriers. Ungroupable strains were not met with.

According to WILLIAMS and MAXTED [15], by T agglutination more than 90 per cent of the strains can be typed. In Hungary MIHÁLYFI and FÜRÉSZ were unable to type by T antigen determination 16-23 per cent of their strains. In our material 84.3 (81.0-89.0) per cent of the strains was determined by agglutination. Most type 19 strains were untypable by agglutination (of our 57 only 7 could be determined in this manner). Type 19 strains produced mostly mucoid or matt colonies.

WILLIAMS and MAXTED [15] could detect the M antigen in 60-80 per cent of their strains; the rest of the cultures grew in the glossy form. On the basis of the M antigen, KÖHLER [16] typed 60 per cent, ROTTA [17] 48 per cent, of the isolated strains. Our data stand very close to the 1960-1961 result of ROTTA, as we typed 48.7 per cent of our strains by M precipitation. The occurrence of such cultures was between 35 and 70 per cent in the different years. The low incidence of strains typable by M precipitation may be explained by the frequent occurrence in our material of the weak anti-M serum producer complexes (5, 11 ... and 8, 25 ...). We have been unable to produce anti-M sera 8, 25 and 27 of acceptable quality. Sometimes during our work we had no anti-M sera 2 and 5 of sufficient titre.

Of the strains 7.5 per cent was untypable by agglutination as well as by precipitation. The high incidence of such strains was attributed to the lack of certain sera.

As to the percentage distribution of prevalent types, ROTTA [17] in 1960 and 1961 noted a more frequent occurrence of types 1, 3, 12 and of complexes 3, 13, B₃₂₆₄ and 5, 11, 12 . . . (7.8—13.3 per cent). KÖHLER [18] in East Germany observed the predominance of type 6 (15 per cent) among 520 strains. CIUCA's examinations performed in Roumania during 1945—1959 revealed the frequency of types 1; 4, 24 . . .; 6; 8, 25 . . .; 11, 12 . . .; 12, and 2. The predominance of type 1 until 1958 is of interest to note, because it is mentioned among the most frequent strains both by STRAUSS [20] in 1950, and by MIHÁLYFI and FÜRESZ [4] in 1949—1953 from Czechoslovakia and Hungary, respectively.

As mentioned above, among our strains complexes 5, 11 . . ., 3, 13, B₃₂₆₄ and 8, 25, Imp. 19 and types 1, 3 and 12 predominated. These data are very similar to those revealed in Czechoslovakia in 1960—1961 [16]. The similarity is explained by the neighbouring geographical situation of the two countries.

As sufficient records could not be obtained, from the present study no conclusions have been drawn as to the association between the serotype of the causative agent and various streptococcal infections.

Although our future work will presumably be improved by the introduction of certain new sera, we hope to have established the basis of a laboratory procedure assisting investigations into the clinical and epidemiological problems of streptococcal infections in Hungary.

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BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

III. UNTERSUCHUNG DER ROLLE DER *PROTEUS VULGARIS*- UND DER *PROTEUS MIRABILIS*-STÄMME

Von

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(Eingegangen am 26. April 1963)

Zusammenfassung. 1. Es wurden 376 an enteraler und 638 an extraenteraler Erkrankung leidenden Säuglinge und Kleinkinder zwecks Klärung der enteropathogenen Rolle der Serotypen von *Proteus vulgaris* und *mirabilis* untersucht.

2. Das Ergebnis der Stuhluntersuchungen zeigte, dass ein grosser Teil der in Krankenhauspflege befindlichen Kranken — ohne Rücksicht auf das Krankheitsbild — über eine spezielle »Krankenhaus« Proteusflora verfügen, die von derjenigen gesunder Personen wesentlich abweicht und die durch das äusserst häufige Vorkommen der zu den *P. mirabilis*-Gruppen O : 3, O : 6, O : 26 und O : 28 gehörenden Stämme charakterisiert ist.

3. Anlässlich der Untersuchung der 376 enteralen Fälle isolierten wir aus dem Stuhl von 98 Kranken *P. vulgaris* und *mirabilis*-Keime. Die Proteus-Stämme kamen in 36 Fällen mit *E. coli dyspepsiae* und Shigella zusammen vor. Die klinische Auswertung der 62 »reinen Proteusfälle«, sowie der Umstand, dass wir aus den Kontakt-Hausinfektionen solche Serotypen isolierten, die bei solchen Kranken gefunden wurden, die längere Zeit hindurch in unserer Abteilung gepflegt worden waren, machen die fakultative Enteropathogenität der Proteus-Stämme wahrscheinlich.

4. Die Antibiotikaempfindlichkeit der Proteus-Stämme entsprach den aus der Literatur bekannten früheren Angaben. Wir haben den Nachweis erbracht, dass die von den Gliedern der anderen Gruppen auffallend abweichende Streptomycinresistenz der in den Krankenhäusern besonders häufigen Stämme O : 3 und O : 26 von der antibiotischen Behandlung der Kranken unabhängig ist.

Die Bakterien *P. vulgaris* und *mirabilis* kommen bekanntlich oft als primäre oder sekundäre Krankheitserreger von extraenteralen Erkrankungen vor. Die Fähigkeit dieser Keime, enterale Erkrankungen hervorzurufen, wurde schon früher angenommen, doch liessen sich bisher mangels mit entsprechenden Methoden durchgeführter Untersuchungen diesbezüglich keine Schlussfolgerungen ziehen. Das Vorkommen der *P. vulgaris*- und *mirabilis*-Stämme in verschiedenem Material bzw. ihre Verteilung in Serotypen wurde von wenigen Autoren untersucht (WINKLE [1], BELYAVIN *et al.* [2], KAUFFMANN [3] und PERCH [6]). Aus ihren Untersuchungen geht jedoch nicht hervor, ob die vom Stuhl isolierten Serotypen von kranken oder gesunden Personen stammten. Auch die Verteilung je nach Alter wurde nicht mitgeteilt. LÁNYI [4, 5] berichtete an Hand seiner 1956 und 1957 durchgeführten Untersuchungen über das Vorkommen der Serotypen *P. vulgaris* und *mirabilis* im Stuhl von in verschiedene Gruppen eingereihten Personen sowie auch im nicht fäkalen Material.

Material und Methoden

Isolierung der Stämme. Die Aufarbeitung des untersuchten Materials und das Sammeln der Stämme geschah mit der in der ersten Mitteilung dieser Serie beschriebenen Technik.

Biochemische und serologische Untersuchungen. Diese wurden in der durch LÁNYI [4] beschriebenen Weise durchgeführt. Eine Ausnahme bildete die Bestimmung der O-Antigene, die statt der Anwendung des die Proteus-Stämme mehr oder weniger hemmenden Desoxycholat-Citrat-Agars mittels einer auf Desoxycholat-Mannit-Nährboden gewachsenen Kultur vorgenommen wurde.

Der Desoxycholat-Mannit-Nährboden hat folgende Zusammensetzung: Pferdefleischsaft 1000 ml, Agar 17 g, Richter-Pepton 10 g, NaCl 5 g, Mannit 10 g, Desoxycholsäure (in Alkali gelöst) 3 g, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ (0,8%ige Lösung) 10 ml, KH_2PO_4 1 g, K_2HPO_4 3 g, Phenolrot (0,2%ige Lösung) 10 ml. Nach Sterilisierung werden dem Nährboden (1000 ml) die folgenden zugesetzt: Ferroammoncitrat (grünschuppige Kristalle) 2 g, Urea 2 g.

Die beiden letzteren Substanzen werden vor dem Zusatz zum Nährboden in je 20 ml Wasser gelöst und die Lösungen zum Kochen gebracht. Das End-pH des Nährbodens beträgt 7,2. Derselbe wird in sterile Röhren abgefüllt und nach RUSSEL geschrägt. Die Beimpfung des Nährbodens wird mittels Stich in den hohen Teil und mit Anscheiben auf den schrägen Teil vorgenommen. Auf dem Nährboden wuchsen die verschiedenen Darmbakterien in folgender Weise:

- *P. vulgaris* und *mirabilis*: der Nährboden ist durchwegs rot mit einem schwarzen Streifen im hohen Teil;
- *P. morganii*: der Nährboden ist durchwegs dunkelrot;
- *P. rettgeri*: im hohen Teil des Nährbodens gelbe Färbung in geringer Ausdehnung, sonst dunkelrot gefärbt;
- *Mannitzersetzende Darmbakterien*: im Nährboden bildet sich zitronengelbe Verfärbung.

Zum Nachweis der H_2S -Bildung verwendeten wir den obigen Nährboden unter Weglassung des Bleiazetat-Agars. Dieser zeigte infolge seines Mannitgehaltes die Verunreinigung der Stämme mit etwaigen sonstigen Darmbakterien in hervorragender Weise. Infolge seines Desoxycholatgehaltes verhinderte dieser Nährboden die Entwicklung von Geisseln und ermöglichte auf diese Weise die Bestimmung der O-Antigene mit Objektträger-Agglutination. Zur Bezeichnung des serologischen Typs der Stämme haben wir das durch KAUFFMANN [3] und PRECH [6] mitgeteilte Schema verwendet.

Die Antibiotikumempfindlichkeit wurde mittels Biotest-Papierstreifen bestimmt («Humán» Institut für Impfstoffproduktion und Forschung, Budapest).

Ergebnisse

Von den 1014 untersuchten Kranken litten 376 an enteralen Symptomen, 638 an sonstigen, extraenteralen Erkrankungen. Das Vorkommen der Serotypen *P. vulgaris* und der verschiedenen *P. mirabilis* in den zwei Untersuchungsgruppen wird in Tabelle I und II verglichen. Diese Tabellen wiedergeben die Verteilung der anlässlich der ersten bakteriologischen Untersuchung gezüchteten Typen.

Wir haben aus dem Stuhl der 1014 Kranken anlässlich der ersten bakteriologischen Untersuchung 318 Proteus-Stämme gezüchtet (32 *P. vulgaris* und 286 *P. mirabilis*). Die *P. vulgaris*-Stämme konnten in mindestens 18, die *P. mirabilis*-Stämme in mindestens 40 unterschiedliche Serotypen eingereiht werden. In Tabelle I und II wurde ausschliesslich die Verteilung der zu den wichtigeren, öfters vorkommenden Gruppen gehörenden Stämme wiedergegeben. Man sieht, dass die biochemischen und serologischen Gruppen im Stuhl der enteralen und sonstiger Kranken im grossen und ganzen eine gleiche

Verteilung aufweisen. Eine Ausnahme bilden die Gruppen *P. mirabilis* O : 26 und O : 6, in den enteralen Fällen nahezu zweimal so oft aufzufinden waren als in den sonstigen. Dagegen kamen die in die Gruppe O : 13 und in sonstige O-Gruppen gehörenden *P. mirabilis*-Keime in den enteralen Fällen seltener vor.

Tabelle I

Angaben von *Proteus vulgaris* und *mirabilis* Serotypen ausscheidenden enteralen Kranken

Gruppe	Zahl der Kranken	Prozentuale Verteilung des Krankengutes	Zahl der Kranken aus denen		Verteilung der Infektionen		Gastro-Enterocolitis (Proteus-F.)		
			Proteus gezüchtet wurde	Proteus und bekannte pathogene Keime gezüchtet wurden	außerhalb des Krankenhauses	innerhalb des Krankenhauses	schwer	mittelschwer	leicht
<i>P. vulgaris</i>	10	10,2	5	5	7 (3)	3 (2)	—	1	4
<i>P. mirabilis</i> O : 3	31	31,7	22	9	23 (5)	8 (4)	2	9	11
<i>P. mirabilis</i> O : 6 ...	7	7,1	5	2	3	4 (2)	1	2	2
<i>P. mirabilis</i> O : 10	6	6,1	5	1	5	1 (1)	—	1	4
<i>P. mirabilis</i> O : 13	2	2,0	1	1	—	2 (1)	—	—	1
<i>P. mirabilis</i> O : 26	18	18,4	10	8	7 (2)	11 (5)	1	2	7
<i>P. mirabilis</i> O : 28	11	11,2	6	5	4 (1)	7 (4)	1	1	4
<i>P. mirabilis</i> sonstige ..	13	13,3	8	5	11 (3)	2 (2)	1	3	4
	98	100,0	62	36	60 (14)	38 (22)	6	19	37

NB. In Klammern ist die Zahl der Fälle verzeichnet, bei denen auch bekannte pathogene Keime wuchsen.

Auffallend ist die Häufigkeit der Gruppe O : 3 (31,6% bzw. 30,8%) in beiden Untersuchungsgruppen. Es sei erwähnt, dass unter den Gliedern der Gruppe O : 3 am häufigsten die unbeweglichen, geissellosen Varianten vorkamen. (Bei den enteralen Kranken waren von 31 Stämmen 25, bei den sonstigen Kranken von 68 Stämmen 58 derartige Varianten.) Die ebenfalls häufigen, mit der Gruppe O : 3 antigenstrukturell verwandten Stämme O : 26 entsprachen dem Serotyp 26 : 3. Letzterer Serotyp, ebenso wie die Gruppen O : 6 kamen

bei enteralen Kranken mehrfach vor, u. zw. aus dem Grunde, weil dieselben sich im Laufe von April und Mai aus den innerhalb des Krankenhauses erworbenen Enteritisfällen häufiger entwickelten. Aus dem Stuhl der enteralen Kranken konnten *Proteus*-Keime anlässlich der ersten Untersuchung in 26,0%, aus dem der sonstigen Kranken in 34,5% gezüchtet werden.

Tabelle II

Angaben von *Proteus vulgaris* und *mirabilis* Serotypen ausscheidenden, an extraenteraler Erkrankung leidenden Kranken

Gruppe	Zahl der Kranken	Prozentuale Verteilung des Krankengutes	Zahl der Kranken, aus denen		Verteilung der Fälle je nach Krankheitsbild				
			Proteus gezüchtet wurden	Proteus und bekannte Keime gezüchtet wurden	a	b	c	d	e
<i>P. vulgaris</i>	22	10,0	20	2	14	1	5	—	2
<i>P. mirabilis</i> O : 3	68	31,0	68	—	27	9	21	4	7
<i>P. mirabilis</i> O : 6	8	3,6	7	1	3	1	3	—	1
<i>P. mirabilis</i> O : 10	12	5,5	12	—	5	—	3	1	3
<i>P. mirabilis</i> O : 13	21	9,5	21	—	7	6	6	1	1
<i>P. mirabilis</i> O : 26	21	9,5	21	—	5	2	6	2	6
<i>P. mirabilis</i> O : 28	16	7,3	16	—	5	1	4	—	6
<i>P. mirabilis</i> sonstige ..	52	23,6	52	—	26	6	9	1	10
	220	100,0	217	3	92	26	57	9	36

a = Grippe

b = Otitis, Mastoiditis

c = Bronchitis, Bronchopneumonie

d = Otitis, Mastoiditis und Bronchitis, Bronchopneumonie zusammen

e = Sonstige Erkrankung

Im Laufe der Untersuchungen wuchsen die *Proteus*-Keime vielfach zusammen mit bekannten Krankheitserregern (*E. coli dyspepsiae*, Shigella, Salmonella). In dieser Hinsicht zeigte sich ein wesentlicher Unterschied zwischen der enteralen und der sonstigen Gruppe; von 98 *Proteus* ausscheidenden Kranken wuchsen bei 36 gleichzeitig auch sonstigen bekannte Krankheitserreger.

Der Unterschied ist dem Umstand zuzuschreiben, dass die bekannten Erreger auch im Verhältnis zu sämtlichen enteralen Kranken viel häufiger waren (von den 376 enteralen Kranken schieden 104, von den 638 enteral symptomfreien insgesamt bloss 8 bekannte Erreger aus).

Aus dem Stuhl der 98 *Proteus* ausscheidenden Kranken isolierten wir anlässlich der ersten Untersuchung in 30 Fällen *Dyspepsie coli* in 6 Fällen Shigellen. Die letzteren abgerechnet, konnten also vom Stuhl der untersuchten 376 enteralen Kranken in 62 Fällen (16,4%) *P. vulgaris* und *mirabilis* ohne bekannte Krankheitserreger gezüchtet werden. Die Schwere der Enteritis oder Enterocolitis wurde in Tabelle I auf die letzteren Fälle bezogen wiedergegeben. Hinsichtlich der Verteilung der Serotypen kommt in den schweren und mittelschweren Fällen auch hier die Gruppe O : 3 am häufigsten vor. Mit Rücksicht auf die Häufigkeit ihres Vorkommens in der Kontrollgruppe kann diese Beobachtung auch eine zufällige sein. Bei der Stuhluntersuchung der Enteritis-Kranken wurde häufig die Erfahrung gemacht, dass die *Proteus*-Kolonien im Verhältnis zu denjenigen der normalen Darmflora in überwiegender Zahl vertreten waren, oder *Proteus* mehr als einmal in Reinkultur wuchs. Der zweite Teil der Tabelle II zeigt die Verteilung der *Proteus*typen je nach Krankheitsbild bei den enteral symptomfreien Kranken. Signifikante Unterschiede sind nicht zu finden. Den Stuhl von insgesamt 16 Kranken untersuchten wir einmal oder wiederholt. Derselbe Serotyp wuchs in 10 Fällen, bei 6 Kranken isolierten wir anlässlich der wiederholten Untersuchungen einen vom ersten abweichenden Serotyp.

Unsere Enteritisfälle lassen sich in zwei Gruppen einteilen, je nachdem die Enteritis bereits vor der Aufnahme bestand oder dieselbe im Krankenhaus erworben wurde. In die erstere Gruppe gehörten 228, in die letztere 148 Kranken. Aus Tabelle I ist ersichtlich, dass bei den im Krankenhaus begonnenen Fällen *Proteus* häufiger zusammen mit *Dyspepsie coli* vorkam, als bei den ausserhalb des Krankenhauses erfolgten Infektionen. Tabelle III zeigt die während des Jahres vorgekommenen Hausinfektionen in monatlicher Verteilung. Es ist hieraus ersichtlich, dass die durch *Proteus*, *Proteus* + bekannte Enteropathogene bzw. ohne *Proteus* durch bekannte Enteropathogene hervorgerufenen Fälle in den Monaten April und Mai eine bedeutende Häufung aufweisen. Diejenigen Hausinfektionen, aus denen in grösserer Zahl *Proteus* O : 26, in kleineren Mengen O : 6-Stämme gezüchtet werden konnten, fallen auf diese Periode. Aus Tabelle III geht klar hervor, dass in April für die Hausinfektionen fast ausschliesslich die bekannten Enteropathogenen und die *Proteus*-Stämme verantwortlich waren. (Von 32 in 31 Fällen.) In Mai kamen diese Keime von 38 in 24 Fällen vor. Bei den übrigen Monaten vorgekommenen Hausinfektionen findet man nicht mehr diese Verteilung. Bei den Hausinfektionen kamen Shigellen verhältnismässig selten vor. Von den 22 Kranken, bei denen *Proteus* mit bekanntem Krankheitserreger gezüchtet wurde, erwiesen sich bloss zwei als *Sh. sonnei*-Ausscheider, aus dem Stuhl der übrigen isolierten wir neben *Proteus* auch *Dyspepsie coli*. Von den 148 Hausinfektionen waren insgesamt 45 durch *E. coli* und 4 durch Shigellen verursachte Erkrankungen. Bezüglich der ausserhalb des Krankenhauses erworbenen Infektionen war die

Tabelle III

Monatliche Verteilung der nosokomialen Gastro-Enterocolitis-Infektionen
(Auf Grund der bei der ersten Untersuchung erfolgten Isolierung)

	M o n a t e												Ins- gesamt
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	
Proteus	—	—	—	6	5	—	—	—	3	—	2	—	16
Proteus + bekannte enteropathogene Keime ...	—	—	—	9	6	1	—	1	3	1	1	—	22
Bekannte enteropathogene Keime	—	—	—	16	13	4	2	1	6	6	1	—	49
Sonstige	1	1	3	1	14	9	4	1	13	12	1	1	61
Gesamtzahl der im Krankenhaus begonnenen Fälle	1	1	3	32	38	14	6	3	25	19	5	1	148

Verteilung der Proteus-positiven Enteritisfälle die folgende: im ersten Quartal 2, im zweiten 23, im dritten 23, im vierten 12. Die überwiegende Mehrzahl der Fälle fiel auf die Periode zwischen Mai und Oktober.

Untersuchungen in bezug auf Antibiotika-Empfindlichkeit. Tabelle IV zeigt das Verhalten der anlässlich der ersten und der wiederholten Untersuchungen isolierten 338 *P. vulgaris* und *mirabilis*-Stämme gegenüber Streptomycin, Chloramphenicol und Tetracycline.

Tabelle IV

Empfindlichkeit der isolierten *Proteus vulgaris*- und *mirabilis*-Stämme
gegenüber Streptomycin, Chloramphenicol und Tetracycline

Zahl der geprüften Stämme: 338

G r u p p e	Zahl der Stämme	Streptomycin		Chloramphenicol		Tetracycline	
		E.	R.	E.	R.	E.	R.
<i>P. vulgaris</i>	39	38	1	39	—	36	3
<i>P. mirabilis</i> O : 3	105	6	99	103	2	—	105
<i>P. mirabilis</i> O : 6	18	18	—	17	1	—	18
<i>P. mirabilis</i> O : 10	18	18	—	18	—	—	18
<i>P. mirabilis</i> O : 13	18	16	2	18	—	—	18
<i>P. mirabilis</i> O : 26	43	1	42	12	31	—	43
<i>P. mirabilis</i> O : 28	29	16	13	25	4	2	27
<i>P. mirabilis</i> , sonstige	68	63	5	62	6	1	67

NB — E. = empfindlich
R. = resistent

Aus Tabelle IV geht hervor, dass ein grosser Teil der *P. vulgaris*-Stämme gegen Tetracycline empfindlich ist, während unter den *P. mirabilis*-Stämmen keine einzige empfindlich war. Gegen Chloramphenicol resistente Stämme kamen hauptsächlich beim Serotyp O:26 vor; von den insgesamt 43 isolierten Stämmen waren 31 resistent. Von den 295 zu sonstigen Serotypen gehörenden Stämmen waren bloss 13 chloramphenicolresistent. Die Empfindlichkeit der Stämme gegen Streptomycin weist je nach Serotyp eine auffallende Differenz auf. Von den 43 *P. mirabilis*-Stämmen O:26 waren 42, von den 105 Stämmen O:3 99 streptomycinresistent. Von den 190 sonstigen Stämmen waren bloss 21 streptomycinresistent. Innerhalb der Gruppe O:3 waren sämtliche geissellose Varianten ohne Ausnahme streptomycinresistent; die empfindlichen Stämme gehörten zu den Serotypen 3:1 und 3:2. Das häufige Vorkommen der Stämme O:3 und O:26 lässt sich mit der Streptomycintherapie nicht erklären. Von den 55 enteralen Kranken, die in die letzteren Gruppen gehörende, gegen Streptomycin resistente Stämme ausschieden, erhielten bloss 8 eine Streptomycintherapie. Zwischen dem Vorkommen sonstiger streptomycinresistenter Serotypen und der Streptomycintherapie konnte ebenfalls kein Zusammenhang nachgewiesen werden. Bei den beiden Untersuchungsgruppen konnten wir bezüglich Antibiotika-Empfindlichkeit keinen Unterschied zwischen den von enteralen und enteral symptomfreien Kranken isolierten *Proteus* Serotypen finden.

In bezug auf die übrigen Antibiotika war die Lage die folgende. Die überwiegende Mehrzahl der Stämme war penicillin- und sulfonamidresistent und es kamen nur einige mässig empfindliche Stämme vor. Gegenüber Polymyxin verhielten sie sich in ähnlicher Weise; von den 338 Stämmen fanden wir bloss 3 empfindliche, die übrigen waren resistent. Alle Stämme waren gegen Erythromycin resistent. Mit Ausnahme von 3 zum gleichen Serotyp gehörenden Stämmen waren sämtliche *P. vulgaris* und *mirabilis* neomycinempfindlich.

Klinische Beobachtungen. Von den 98 enteralen Kranken beschränkten wir uns auf die Schilderung der klinischen Angaben derjenigen 62 Fälle (46 Säuglinge und 16 Kinder), aus deren Stuhl sich *Proteus* ohne bekannte Krankheitserreger züchten liess. Unser Krankengut wurde auf Grund der enteralen Symptome und des Krankheitsbildes in drei Gruppen u. zw. schwere, mittelschwere und leichte Gastroenteritis, Enteritis bzw. Enterocolitis eingeteilt (Tabelle I).

Symptome von schwere Gastroenteritis wurden bei 6 Kranken beobachtet. Charakteristisch war die hohe Temperatur (39–41 °C), täglich 6–8mal Erbrechen und 8–12 dünne, schleimige, wässrige, zuweilen explosionsartige Stuhlentleerungen, hochgradiger Verfall, Eklampsie, Sensoriumstörung und Gewichtssturz. Bei zwei Kranken konnten wir die Ausbildung toxischer Symptome beobachten, einen von ihnen (4½ Jahre alt) haben wir verloren. Zum gastroenteralen Krankheitsbild gesellte sich in keinem Fall in extra-

enteraler Prozess oder sonstige Erkrankung hinzu. Die Dauer der Krankheit betrug vom Auftreten der Symptome bis zur klinischen Ausheilung 5–18 Tage. Zu dieser klinischen Gruppe gehörten 4 Säuglinge (1–8 Monate alt) und 2 Kinder ($3\frac{1}{2}$ bzw. $4\frac{1}{2}$ Jahre alt). Von den Säuglingen waren zwei mit Muttermilch ernährt und zwei auf künstliche Nahrung eingestellt.

Mittelschwere Gastroenteritis bzw. Enterocolitis kam in 19 Fällen zur Beobachtung. Das Krankheitsbild war charakterisiert durch Fieber von 38 bis 39 °C, seltener bis 40 °C, täglich 2–3 mitunter noch mehrmals Erbrechen, 8–10 flüssige, schleimige, wässrige, bei 2 Kranken blutige, häufig übelriechende Stühle, demzufolge Gewichtsabnahme, Appetitlosigkeit, Mattigkeit, schlechter Allgemeinzustand. Sechs Kranken behandelten wir wegen der sich zu der extraenteralen Grundkrankheit (Otitis, Mastoiditis, Bronchopneumonie) gesellenden Gastroenteritis, 13 Kranken auf Grund ihrer rein enteralen Symptome. Während der 6–25 (durchschnittlich 14) Tage dauernden Krankenhauspflge wurden die Kranken symptomfrei. Hinsichtlich Altersverteilung gehörten ins I.–II. Trimenon 11, ins III.–IV. Trimenon 6 und in die Krankheitsgruppe über ein Jahr (13 bzw. 16 Monate alt) 2 Kranken. Fünf Säuglinge waren auf Muttermilch, 13 erhielten künstliche Nahrung.

Mit leichter Gastroenteritis bzw. Enterocolitis befanden sich 37 Kranken in unserer Behandlung. Die Symptome der zu dieser Gruppe gehörenden Kranken waren Subfebrilität bzw. Fieber um 38°, Mattigkeit, Appetitlosigkeit. Bei den gastroenteralen Krankheitsformen waren täglich 1–3mal Erbrechen, täglich 6–8 schleimige, flüssige, wässrige, zumeist übelriechende Stühle zu beobachten. Bei Säuglingen zeigte die Gewichtskurve eine stillstehende oder verflachende Tendenz. Ein rein enterales Krankheitsbild wurde in 27 Fällen, ein solches mit extraenteralem Prozess kompliziert (Grippe, Bronchitis, Mastoiditis, Cystitis) in 10 Fällen beobachtet. Der durchschnittliche Krankheitsverlauf betrug 3–10 Tage; eine sich hinziehende Krankheitsform (20–25 Tage) konnte bei 2 Kindern beobachtet werden. Die Verteilung der 37 Kranken je nach Alter: Säuglinge (hievon 16 im I.–II. Trimenon, 9 im III.–IV. Trimenon), 12 Kinder (zwischen 1–2 Jahren). Vier Säuglinge waren muttermilchernährt, 3 erhielten gemischte und 18 künstliche Nahrung.

Die Kranken wurden mit Sulfonamid, Streptomycin, Tetracyclin, Furadantin, Polymyxin B, i. m., ferner mit Chloramphenicol, Neomycin und Erythromycin peroral behandelt. Nach der Züchtung der für pathogen gehaltenen Stämme wurde die Therapie der Antibiotikaempfindlichkeit entsprechend fortgesetzt. Die symptomatische Behandlung bestand aus der Verabreichung von Ringer-Dextrose, Plasma- und Bluttransfusionen, Polyvitaminen und Gamma-Globulin. Ein Kind von $4\frac{1}{2}$ Jahren starb, die anderen Patienten wurden alle klinisch symptomfrei.

Von den 62 Kranken gelangten 46 mit enteralen Beschwerden in unsere Abteilung, in 16 Fällen entwickelte sich das gastroenterale Krankheitsbild

nach der Aufnahme ins Krankenhaus, diese letzteren sind demnach innerhalb des Krankenhauses infiziert worden. Von einer ausgesprochenen Proteus-Epidemie handelte es sich nicht, obwohl in gewissen Monaten (z. B. April-Mai) die Zahl der durch Proteus O:3, O:6, O:26 verursachten Gastroenteritisfälle ein gehäufteres Vorkommen zeigte.

Besprechung

In bezug auf das Vorkommen der *P. vulgaris* und *mirabilis*-Stämme geht aus den von LÁNYI 1956 mitgeteilten Angaben hervor, dass diese vorwiegend im Stuhl der in Krankenhäusern behandelten Säuglingen zu finden waren (von 585 Fällen 205 positiv, 35,0% [4]). Im Stuhl der als Kontrolle untersuchten 102 zweifellos gesunden, weder an enteraler noch an sonstiger Krankheit leidenden Säuglingen waren diese Keime in 12,7% zu finden. Im Stuhl gesunder Erwachsenen waren dagegen *P. vulgaris* und *mirabilis* viel seltener zu beobachten (von 7419 Fällen in 197 = 2,6%). Bezüglich ihrer Verteilung nach Serotypen war unter den im Krankenhaus gepflegten Säuglingen die Häufigkeit der in die Gruppen O:3 und O:26 gehörenden Stämme auffallend (32,1% bzw. 20,6%). In jedem der untersuchten 5 Budapester Krankenhäuser waren besonders die Glieder der Gruppe O:3 zu isolieren. Im Stuhl der gesunden Säuglinge sind diese Serotypen überhaupt nicht vorgekommen, bei gesunden Erwachsenen war ihre Häufigkeit im Verhältnis zu den übrigen Typen 3,0% bzw. 2,5%. Bei urogenitalen und otologischen Erkrankungen kamen diese 2 Gruppen im Verhältnis zu den übrigen *P. vulgaris* und *mirabilis*-Stämmen in 32,1% bzw. 20,6% vor [5].

Vergleichen wir unsere vorliegenden Untersuchungen mit den obigen Angaben, so ist die Ähnlichkeit der Ergebnisse auffallend. Aus dem Stuhl der enteralen und sonstiger Kranken liessen sich diesmal *P. vulgaris* und *mirabilis* in 26,0% bzw. 34,5% züchten. Auch die Verteilung der Serotypen weist eine grosse Ähnlichkeit auf, die Stämme O:3 und O:26 sind auch hier häufig und auch die Verteilung der übrigen Serotypen stimmt im grossen und ganzen überein. Aus unseren gegenwärtigen Ergebnissen geht eindeutig hervor, dass unter den in unserem Krankenhaus gepflegten Säuglingen — ohne Rücksicht darauf, an welcher Krankheit sie litten — die Proteus-Stämme weit verbreitet waren. Auf Grund der Obigen kann mit Recht der Schluss gezogen werden, dass — mindestens die in Krankenhäusern gepflegten kranken Säuglinge — über eine spezielle Proteusflora verfügen, die sowohl bezüglich der Häufigkeit des Vorkommens als auch in bezug auf Verteilung der Serotypen sich von der Proteusflora gesunder Erwachsenen unterscheidet. Die durch LÁNYI geprüften 102 Fälle zwecks Ermittlung der Proteusflora

der gesunden Säuglinge scheinen etwas gering zu sein, doch wenn man dies als Basis annimmt, so findet man auch hier im Verhältnis zu den in Krankenhauspflege befindlichen Säuglingen einen Unterschied.

Die bei enteral symptomfreien Kranken beobachtete ähnliche Typenverteilung und das häufige Vorkommen des *P. vulgaris* und *mirabilis* bei diesen, scheint die Fähigkeit dieser Keime, enterale Symptome hervorzurufen, zu verneinen. Aber in Anbetracht dessen, dass vom Stuhl der 376 enteralen Kranken in 62 Fällen (16,4%) ausser *Proteus* kein sonstiger Erreger zu isolieren war, weiters, dass aus dem Fäzes der enteritischen Kranken der *Proteus* im Verhältnis zu den übrigen Darmbakterien oft in überwiegender Mehrzahl, ja sogar in Reinkultur wuchs, muss dem *Proteus* doch eine gewisse enteropathogene Rolle zugeschrieben werden. Auch aus den im Krankenhaus in den Monaten April und Mai vorgekommenen gehäuften, nosokomialen Enteritisfällen liessen sich die *Proteus*-Stämme mehrmals selbständig isolieren.

Die Beobachtung, dass es uns gelang, aus nosokomialen Kontaktinfektionen die häufigsten Serotypen der seit längerer Zeit in unserer Abteilung liegenden Kranken zu isolieren, und mit dem Krankheitsbild in ursächlichen Zusammenhang zu bringen, weist ebenfalls auf eine gewisse enteropathogene Rolle der *Proteus*-Stämme hin. Die enteropathogene Rolle der *Proteus*-Serotypen beweist auch die Tatsache, dass die auf Grund der in vitro Empfindlichkeit der Stämme eingeführte Antibiotikatherapie zur klinischen Symptommfreiheit und zum Aufhören der Bakterienausscheidung führte.

Das gefährdete Alter ist, wie dies aus unseren Daten hervorgeht, das Säuglingsalter, doch kann auch in den Altersgruppen über ein Jahr mit einer *Proteus*infektion gerechnet werden. Die Darmflora stabilisiert sich scheinbar erst im späteren Kindesalter, so dass Qualität und Zusammensetzung der Nahrung (Diätfehler) oder sonstige Faktoren das labile Gleichgewicht der Darmflora der Säuglinge leicht beeinträchtigen können. Darauf verweist auch unsere sich auf Jahre erstreckende Beobachtung, wonach in der Darmflora, namentlich der Säuglinge, ferner der Kinder von 1—2 Jahren, auch solche Gruppen von Darmbakterien häufig und gemischt zu finden sind (*P. morganii*, *P. vulgaris* und *mirabilis*, *Ps. aeruginosa*, *Staph. aureus*), die aus dem Stuhl von Kindern über 2 Jahren seltener gezüchtet werden können. Auch von Darminfektionen werden überwiegend die auf gemischte und künstliche Nahrung gehaltenen Säuglinge befallen. Diese Beobachtung wird auch durch die Angaben unseres vorliegenden Materials unterstützt. Von den 46 gastroenteritischen Säuglingen erhielten 32 künstliche, 3 gemischte Nahrung während 11 muttermilchernährt waren.

Die klinischen Symptome, der Verlauf der enteralen Krankheitsformen im Säuglings- und Kindesalter führen ohne Hilfe der bakteriologischen und serologischen Diagnostik nur selten zur sicheren Erkennung einer durch eine bestimmte enteropathogene Bakteriengruppe verursachten Krankheit (z. B.

E. coli dyspepsiae, Shigella, Salmonella usw.). Im Falle von gemischter Isolierung der bekannten enteralen Krankheitserreger — was auch bei den Gastroenteritiden des Säuglingsalters nicht selten ist — ist es besonders schwer, die charakteristischen Symptome abzugrenzen und den dieselben hervorruufenden ätiologischen Faktor zu beurteilen. Von den bereits erwähnten 98 Kranken konnten wir aus dem Stuhl von 36 Kranken neben Proteus-Stämmen auch *E. coli* Serotypen und Shigellen isolieren. Den oben Gesagten gemäss stossen wir auf Schwierigkeiten, in Verbindung mit diesen Fällen bezüglich der Proteus-Ätiologie Schlüsse zu ziehen. Soviel ist jedenfalls festzustellen, dass ähnlich den Dyspepsien vermutlich reiner Proteus Ätiologie es auch in diesen Fällen gelang, aus dem Stuhl grösstenteils in die Serotypen O:3, O:6, O:26 und O:28 einzuordnende, oft vorkommende Proteus-Stämme nachzuweisen.

Unsere klinischen Beobachtungen lassen auf das Vorkommen solcher Darmbakteriengruppen in den Gastroenteritiden des Säuglings- und Kindesalters schliessen, denen als kommensalen bisher wenig Aufmerksamkeit geschenkt wurde. Die gesammelten Erfahrungen werden — analog zu den Coli-Dyspepsien — auch durch die Tatsache bekräftigt, wonach die Zahl der bakteriologisch nicht verifizierbaren Darminfektionen des Säuglings- und Kindesalters noch immer beträchtlich ist. Auf Grund der Summierung der klinischen und bakteriologischen Beobachtungen können gewisse Proteus-Serotypen als fakultative enteropathogene Keime des Säuglingsalters betrachtet werden.

Besondere Aufmerksamkeit verdient die abweichende Antibiotikaempfindlichkeit der einzelnen Proteustypen. Das Verhalten der *P. vulgaris* und *mirabilis* gegenüber Tetracycline entspricht demjenigen, das PERCH [6] sowie LÁNYI [5] in ihren früheren Untersuchungen gefunden haben. Die von den übrigen Serotypen abweichende Streptomycinresistenz der serologisch verwandten Stämme O:3 und O:26 wurde von LÁNYI 1957 nachgewiesen. Die Situation hat sich auch seither nicht geändert, da ja unter den Gliedern der sonstigen Serotypen streptomycinresistente Stämme bloss in minderer Zahl vorkamen. Im Laufe unserer gegenwärtigen Untersuchungen bestätigte es sich, dass zwischen dem Vorkommen der *P. mirabilis*-Gruppen O:3 und O:26 und der Behandlung der einzelnen Kranken mit Streptomycin kein Zusammenhang besteht. Da nach PERCH [6] die Streptomycinempfindlichkeit der vor dem Antibiotikazeitalter isolierten Proteus-Serotypen voneinander nicht abweicht, muss angenommen werden, dass im Gegensatz zu anderen Typen die Glieder der erwähnten zwei Antigengruppen mehr Gelegenheit hatten, sich eine Resistenz zu verschaffen, sei es aus endogenen Gründen, sei es aber als in Krankenhäusern besonders häufig vorkommende »Hausstämme«. Der letztere Umstand scheint viel mehr an der Hand zu liegen, und dies scheinen einzelne noch nicht veröffentlichte Untersuchungen [7]

zu bestätigen, wonach das Mutationsverhältnis der in die streptomycinempfindliche Gruppe O:3 gehörenden Stämme sich von demjenigen anderer Serotypen nicht unterscheidet.

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BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

IV. UNTERSUCHUNG DER ROLLE DER PROTEUS MORGANII-STÄMME

Von

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Zusammenfassung. 1. Es wurden aus den Stuhlproben von 240 Säuglingen und Kindern 257 *P. morganii*-Stämme isoliert.

2. Mit Hilfe von 29 O-Gruppen-Seren konnten wir 212 Stämme (82%) einer der O-Gruppen zuordnen.

3. Wir beobachteten das Vorkommen von 11 verschiedenen O-Antigenen. Die meisten Stämme gehörten in die erste O-Gruppe (163; 78%). Von den übrigen verdienen die O : 2, O : 4 und O : 16 erwähnt zu werden.

4. Hinsichtlich der Serogruppenverteilung der aus enteralen und Kontrollfällen gezüchteten Stämme, wie auch hinsichtlich des Verhältnisses der nicht definierbaren Stämme in diesen zwei Gruppen konnten wir keinen Unterschied beobachten.

5. Die hochgradige Antibiotikatoleranz der Stämme war auffallend. Am wirkungsvollsten erwies sich Neomycin (99%).

6. Auf Grund der Ergebnisse unserer Beobachtungen ist eine ätiologische Rolle der *P. morganii*-Stämme in der Enterocolitis der Säuglinge und Kinder nicht als wahrscheinlich anzusprechen.

Seit der ersten Schilderung von MORGAN beschäftigten sich zahlreiche Mitteilungen mit den biochemischen serologischen Einheiten, der systematischen Einordnung und Pathogenität des Morganschen Bacillus *P. morganii*, doch ist es nicht gelungen, diese Fragen völlig zu klären [1—7]. RAUSS stellte 1936 fest, dass dieser Keim auf Grund seiner Propagation und Antigenstruktur zur Proteus-Gruppe gehört [8]. Seine Beobachtungen wurden durch SEVIN und BUTTIAUX [9] verstärkt und von BERGEY's Manual akzeptiert. RAUSS konstatierte auch, dass *P. morganii* in bezug auf seine Antigenstruktur heterogen ist. Auch COWAN [10] steht auf dem Standpunkt, dass dieser Keim zur Proteus-Gruppe gehört. KAUFFMANN [11] betrachtete ihn in seinem Handbuch »Enterobacteriaceae« als einen der vier Spezies des Genus Proteus. Nach der Ansicht von FULTON [12] ist der Keim ein selbständiges Genus: Morganella. KAUFFMANN [13] hob ihn auf Grund von biochemischen Gesichtspunkten aus dem Genus Proteus heraus und teilte ihn unter dem Namen Morganella als selbständiges Genus ein. Nach der Ansicht von EWING [14] muss dieser Keim unter dem Namen Morganella als neues Genus betrachtet werden, mit den Species *Morganella morganii*, *Morganella rettgeri* und *Morganella inconstans* (Providencia). Die Auffassung von RAUSS entspricht derjenigen von KAUFFMANN; er behauptet folgendes: Innerhalb der Proteus-

Gruppe bestehen drei Untergruppen, u. zw. *P. hauseri* (*vulgaris*, *mirabilis*), *P. rettgeri* und *P. morganii* auch *Morganella* genannt [15]. RAUSS teilte 48 Stämme auf Grund ihres O-Antigens in 17 Gruppen ein. Innerhalb der ersten O-Gruppe separierte er drei Untergruppen. Darüber hinaus wies er im Interesse der Typenbestimmung das Vorhandensein von sieben verschiedenen H-Antigenen nach. LEWIS [16] konnte 73% von 242 Stämmen mit Hilfe von 16 Seren bestimmen. Neuerdings teilte SORMOLINSKY [17] seine 110 Stämme mittels fünf Seren in fünf Gruppen ein. RAUSS und VÖRÖS [15] ordneten 222 Stämme in 29 O-Gruppen und auf Grund von 19 H-Antigenen in 57 Serotypen ein. Die erste Gruppe war in drei Untergruppen unterteilt und umfasste 54% der Stämme. In die ersten fünf Gruppen gehörten 70% der von ihnen untersuchten Stämme.

Zweck unserer vorliegenden Untersuchungen war, auf Grund der Bestimmung der O-Antigene der Stämme eine Antwort auf die Frage zu erhalten, ob *P. morganii*-Stämme in der Ätiologie der im Säuglings- und Kindesalter auftretenden Gastroenterocolitiden unbekannter Herkunft eine Rolle spielen können.

Material und Methoden

Stämme. Im Laufe der Aufarbeitung der Stuhlproben der in unserer Spitalsabteilung behandelten Säuglinge und Kinder auf üblichen Plattennährböden (Blutagar, Eosinmethylenblau, Desoxycholat-Citrat, Bismutsulfit-Agar) isolierten wir 257 Stämme. Für das Wachstum der Stämme erwiesen sich Blutagar- und Desoxycholat-Citrat-Platten als die geeignetsten.

Biochemische Reaktionen. Das Verhalten unserer Stämme wurde zunächst auf dem NÓGRÁDY—RODLERSchen Polytrop-Nährboden [18] untersucht, danach nahmen wir die biochemischen Reaktionen auf Grund der von KAUFFMANN [11] angegebenen Standard-Methoden vor. Im Interesse der biochemischen Identifizierung hielten wir folgende Tests für notwendig: Ureum-Abbau; Indol- und H_2S -Bildung; Phenylalanin-Desaminase-Test; Wachstum in KCN Peptonwasser; Gelatineverflüssigung; Citratutilisation; Voges-Proskauersche Reaktion; Methylrot-Test. Hinsichtlich Kohlenhydratfermentation prüften wir den Abbau von Saccharose, Lactose und Dextrose.

Serologie. Die Serogruppen der Stämme (O) haben wir mit Hilfe von Objektträgeragglutination mittels der durch uns gewonnenen diagnostischen Kaninchenimmunsereen geprüft. Wir agglutinierten die Stämme zunächst durch polyvalente Serummischungen [1—7], dann durch entsprechende monovalente Seren. Die Zusammensetzung der polyvalenten Serummischung (Mischverhältnis usw.) haben wir früher [15] mitgeteilt.

Prüfung der Antibiotikaempfindlichkeit. Die Antibiotikaempfindlichkeit der Stämme wurde mittels Biotest-Scheiben auf Grund der Diffusionsmethode durchgeführt (»HUMAN« Institut für Impfstoffproduktion und Forschung, Budapest).

Ergebnisse

Zur Charakterisierung der biochemischen Aktivität der Stämme können folgende Eigenheiten erwähnt werden: Urease-Bildung; Indolproduktion; Mangel der H_2S -Bildung und der Gelatineverflüssigung; positiver Phenylalanin-Desaminase-Test; Wachstum in KCN Peptonwasser; negative Voges-Proskauersche und positive Methylrot-Reaktion. Die Stämme bauten Saccharose im allgemeinen nicht, Lactose nie ab.

Im Laufe der biochemischen Teste zeigten unsere sich für *P. morganii* erwiesenen 257 Stämme die in der Tabelle I wiedergegebene Fermentationsaktivität.

Tabelle I

Fermentations-Untersuchungen der isolierten 257 P. morganii-Stämme

	Positiv	Negativ
Urease	257	—
Indol	257	—
Phenylalanin	257	—
Dextrose	257	—
KCN	247	10
Gelatinase	1	256
Citrat	7	250
Voges-Proskauer	5	252
Methylrot	252	5
Saccharose	7	250
H ₂ S	—	257
Lactose	—	257

Die biochemischen Eigenheiten der Stämme stimmten mit unseren bisherigen Angaben und mit den in der Literatur auffindbaren Daten überein.

Im Laufe der serologischen Identifizierung liessen sich von den mittels 29 O-Gruppenserien untersuchten 257 *P. morganii*-Stämme 212 (82,4%) in einer der Gruppen einordnen. 45 Stämme (17,6%) konnten nicht in Gruppen eingeordnet werden. Von diesen letzteren agglutinierten 11 spontan. Die serologische Gruppenverteilung der eingeordneten *P. morganii*-Stämme ist in Tabelle II wiedergegeben.

Wir beobachteten das Vorkommen von 11 unterschiedlichen O-Antigenen. Aus Tabelle I geht hervor, dass das Gruppenantigen O:1 am häufigsten war, es enthielt etwa 78% der klassifizierbaren Stämme, während die übrigen O-Gruppen nur in kleiner Zahl anzutreffen waren; von diesen sollen bloss O:2, O:4 und O:16 erwähnt werden. Ein Teil der Stämme stammte aus dem Stuhl von 89 an enteralen Symptomen leidenden Säuglingen (106 Stämme), den anderen Teil isolierten wir aus dem Stuhl von 151 an extraenteralen Erkrankungen (Grippe, Pneumonie, Otitis) leidenden Säuglingen und Kinder (151 Stämme). Von den von enteralen Kranken stammenden 106 Stämmen haben wir 88 (83,0%) in Gruppen eingeteilt, während es nicht gelang, die Zugehörigkeit von 18 Stämmen (17,0%) zu bestimmen. Bezüglich der in enteraler Hinsicht symptomfreien Kontrollgruppe konnten wir folgendes beobachten:

Einer serologischen Gruppe liessen sich 124 Stämme (82,1%) zuordnen, während 27 Stämme mit Hilfe der O-Seren nicht zu bestimmen waren. In bezug auf die Gruppenverteilung der Stämme kann festgestellt werden, dass sowohl bei der Kranken-, als auch bei der Kontrollgruppe das Verhältnis der in eine serologische Gruppe einreihbaren und der nicht definierbaren (ND) Stämme sozusagen identisch ist.

Tabelle II

Serologische Gruppenverteilung der isolierten 257 P. morganii-Stämme

Serologische O-Gruppe	Enterale Kranken (aus dem Stuhl)		Kontrollen (aus dem Stuhl)		Insgesamt	
	Zahl	%	Zahl	%	Zahl	%
1	68	77,2	95	76,0	163	77,0
2	6	6,8	10	8,0	16	7,5
4	5	5,7	3	2,5	8	3,0
5	1	1,1	—	—	1	0,5
6	—	—	1	0,8	1	0,5
8	1	1,1	3	2,5	4	1,9
10	2	2,3	1	0,8	3	1,4
11	—	—	3	2,5	3	1,4
13	1	1,1	3	2,5	4	1,9
16	3	3,5	3	2,5	6	2,9
19	1	1,1	2	1,7	3	1,9
In Typen einge- reicht	88		124		212	
ND* Stämme	18		27		45	

*ND = nicht definierbar

Im Laufe der Prüfung der Antibiotikaempfindlichkeit in vitro der isolierten 257 *P. morganii*-Stämme erwiesen sich gegenüber Penicillin 6,2%, gegen Sulfonamide 8,5%, gegen Streptomycin 50,2%, gegen Chloramphenicol 35,0%, gegenüber Chlortetracyclin 53,3, gegen Oxytetracyclin 54,4%, gegen Neomycin 99,2%, gegen Erythromycin 12,8%, gegen Bacitracin 0,8% und gegen Polymyxin B 5,4% der Stämme empfindlich oder mässig empfindlich.

Wie aus den Angaben der Tabelle II hervorgeht, erwies sich von den üblichen Antibiotika Neomycin als das wirksamste, da bloss zwei Stämme eine Resistenz gegen das letztere aufwiesen. Sonst ist die hochgradige Antibiotikatoleranz der Stämme auffallend.

Auf Grund der Antibiotogramme der Stämme konnten wir keine wesentlichen Unterschiede zwischen den Kranken- und Kontrollgruppen beobachten.

Tabelle III*Antibiotikaempfindlichkeit der isolierten 257 P. morganii-Sämme*

Antibiotika	Empfindlich		Resistent	
	Zahl	%	Zahl	%
Penicillin	16	6,2	241	93,8
Sulfonamide	22	8,5	235	91,5
Streptomycin	129	50,2	128	49,8
Chloramphenicol	90	35,0	167	65,0
Chlortetracyclin	137	53,3	120	46,7
Oxytetracyclin	140	54,4	117	45,6
Neomycin	255	99,2	2	0,8
Erythromycin	33	12,8	224	87,2
Bacitracin	2	0,8	255	99,2
Polymyxin B	14	5,4	243	94,6

Tabelle IV*Antibiogramm-Typen der aus der Kranken- und Kontroll-Gruppe isolierten 163 P. morganii O : 1*

Antibiogrammtypen	O : 1 Stämme der Kranken	O : 1 Stämme der Kontrollen
1.	6	17
2.	1	1
3.	37	44
4.	1	11
5.	2	2
6.	—	2
7.	—	2
8.	8	2
9.	—	1
10.	—	1
11.	—	1
12.	—	1
13.	9	10
14.	1	—
15.	1	1
16.	1	—
17.	1	—

Die aus enteralen Kranken isolierten Stämme O:1 liessen sich in 11, die aus der Kontrollgruppe isolierten in 13 Antibiotogrammtypen einteilen. Bei beiden Gruppen dominierten drei Antibiotogrammtypen (1, 3, 13) der Stämme O:1

Klinische Beobachtungen. Von den in unserer Krankenhausabteilung untersuchten 1014 Kranken haben wir in Verbindung mit 240 Krankenhaussfällen, also in 23,6% des ganzen Krankengutes *P. morganii*-Stämme gezüchtet. Von den beobachteten 240 Säuglingen und Kindern bestanden bei 89 enterale, bei 151 extraenterale Symptome. Das Vorkommen der aus dem Stuhl der 89 enteral erkrankten und der 151 enteral symptomfreien (Kontroll) Säuglinge und Kinder isolierten *P. morganii*-Stämme prüfend, erhielten wir sowohl beim enteralen Krankengut (376 Fälle) als auch beim Kontrollmaterial (638 Fälle) das gleiche Ergebnis (23,6%).

Aus den Stuhlproben der 89 an Gastroenterocolitis leidenden Kranken isolierten wir — neben *P. morganii* — in 29 Fällen bekannte enteropathogene Keime (*E. coli dyspepsiae*, Shigella) in 60 Fällen sonstige fäkale Stämme (*E. coli*, Klebsiella, Proteus, *Ps. aeruginosa* oder Staphylococcus). Eine Reinkultur von *P. morganii*-Stämmen konnte nicht beobachtet werden. Die enteralen Symptome von 58 Kranken waren auf eine Infektion vor ihrer Aufnahme zurückzuführen, während 31 Kranken innerhalb des Krankenhauses infiziert wurden. Der ätiologische Faktor der Hausinfektionen war in 15 Fällen *E. coli* O:111 und O:55. Bei 14 ausserhalb des Krankenhauses infizierten Kranken wurden *E. coli dyspepsiae* bzw. Shigella gezüchtet. Bei den ausserhalb des Krankenhauses angesteckten sporadischen und bei den innerhalb der Abteilung infizierten Kontakt-Enterocolitiskranken ergab die beim Auftreten der Symptome durchgeführte erste Stuhluntersuchung bloss in 52 Fällen *P. morganii*; aus dem Stuhl von 37 Kranken konnten die Keime erst später nachgewiesen werden. Laut unseren bei wiederholten Stuhluntersuchungen gemachten Beobachtungen liessen sich im Verlauf der Enterocolitiden der Säuglinge aus den Stuhlproben des gleichen Säuglings nicht selten in verschiedenen Zeitpunkten immer andere Serogruppen des Stammes isolieren. Die aus dem Stuhl der an Gastroenteritis erkrankten, aber enteral symptomfreien Kranken gezüchteten *P. morganii*-Stämme waren im Verhältnis zu den die Fäkalflora bildenden Darmbakterien in minderer Zahl aufzufinden und ihre Isolierung war in vielen Fällen nur aus dem selektiv wirkenden DC-Nährboden möglich.

Von unseren 89 enteralen Kranken waren 65 Säuglinge, davon 40 Kranken 1—6 Monate, 24 über 1 Jahr alt. Ähnlich war die Altersverteilung in der 151 Fälle zählenden Kontrollgruppe. Die Züchtbarkeit der *P. morganii*-Stämme aus dem Stuhl ist auf Grund der Angaben unseres gegenwärtigen Untersuchungsmaterials, aber auch nach unseren sich auf Jahre erstreckenden Beobachtungen im Säuglingsalter am häufigsten und diese vermindert sich allmählich mit dem Fortschreiten des Lebensalters. Isolierungen dieses Keimes sind auch aus dem Stuhl gesunder Neugeborener (2—3 Tage alt) nicht selten.

Tabelle V wiedergibt das Vorkommen des bei 240 kranken und Kontroll-Säuglingen bzw. Kindern anlässlich der ersten Untersuchung aus dem Stuhl isolierten *P.morganii* in monatlicher Verteilung.

Nach den Angaben der Tabelle V lässt sich eine Bakterienausscheidung bei der Mehrzahl der enteralen Kranken im Laufe der Frühjahrs- und Herbstmonate nachweisen. Die häufigere Isolierbarkeit der *P. morganii*-Stämme kann

Tabelle V

*Verteilung der aus 240 Krankenhausfällen isolierten
P. morganii-Stämme je nach Monaten (erste Isolierung)*

Monat	Kranken	Kontrolle	O : 1 Gruppe	Sonstige Gruppen
I.	1	3	—	4
II.	—	18	11	7
III.	1	25	20	6
IV.	11	25	26	10
V.	11	14	17	8
VI.	9	15	12	12
VII.	3	9	7	5
VIII.	4	2	5	1
IX.	14	16	14	16
X.	24	9	25	8
XI.	9	6	9	6
XII.	2	9	7	4
Insgesamt	89	151	153	87

jedoch mit den während dieser beiden Jahreszeiten gehäuft auftretenden, durch *E. coli dyspepsiae* und *Shigella* verursachten Darminfektionen und der Anwuchs des enteralen Krankenmaterials im Krankenhaus in Zusammenhang gebracht werden.

Diese Feststellung scheint um so wahrscheinlicher, da bei den enteral symptomfreien Kontroll-Säuglingen *P. morganii* in jedem Monat des Jahres auch in den vom Gesichtspunkt des Vorkommens der Darminfektionen für ruhig geltenden Perioden isoliert werden kann. Auch die $\frac{2}{3}$ der Stämme bildende Gruppe O:1, die aus dem Stuhl der enteralen und extraenteralen Kranken in gleichem Verhältnis gezüchtet werden kann, zeigt ohne Rücksicht auf die Jahreszeit ein gleichmässiges Vorkommen.

Die Rolle der *P. morganii*-Stämme ausser acht lassend, haben wir bei unseren an Gastroenterocolitis leidenden Kranken die auf Grund der in vitro Antibiotikaempfindlichkeit der enteropathogenen Keime, oder mangels der-

selben des aus dem Stuhl gezüchteten vermutlichen Erregers (*Proteus*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*) gewählte Antibiotikatherapie eingeleitet. Unter der Einwirkung der adäquaten bzw. der als adäquat angenommenen Antibiotikatherapie wurden alle unsere Patienten in 6–14 Tagen symptomfrei. Wir konnten auch anlässlich der nach Ablauf der klinischen Symptome durchgeführten bakteriologischen Stuhluntersuchungen in 30% der Fälle *P. morganii* züchten. Die oben Gesagten werfen ein Licht auf die überaus starke Verbreitung der *P. morganii*-Stämme in Säuglings- und Kleinkinderkollektiven und weisen unserer Ansicht nach gleichzeitig auch auf ihren saprophyten Charakter hin.

Besprechung

Die im Laufe der Untersuchungen gewonnenen Daten bewertend, können wir diejenigen Beobachtungen früherer Autoren bestätigen, wonach zur biochemischen Charakterisierung der Stämme Urease-, Indol-, H_2S -, KCN-, Gelatinase-, Methylrot-, Voges-Proskauer-, Phenylalanin-desaminase-Teste, ferner Lactose-Dextrose- und Saccharose-Fermentationsverfahren geeignet sind. Das durch RAUSS und VÖRÖS ausgearbeitete Antigeneschema und Objektträger-Agglutination sind zur serologischen Typusbestimmung leicht und gut anwendbar.

Als Ergebnis der serologischen Untersuchungen, ähnlich wie in den Beobachtungen von RAUSS und VÖRÖS, ist im Stuhl sowohl der kranken als auch der gesunder Fälle die Dominanz der Serogruppe O:1 von *P. morganii*, nebst des relativ seltenen Vorkommens sonstiger Serogruppen zu beobachten. Die hochgradige Antibiotikatoleranz und der Antibiotigrammtyp der aus der Gruppe der enteralen Kranken und der Kontrollen isolierten, über O:1, aber auch über andere O-Antigene verfügenden Stämme zeigt keine Verschiedenheit. Die parallele Analyse der klinischen und Laboratoriumsangaben weist auf manche Eigenheiten in bezug auf das Vorkommen der Stämme und auf ihre Rolle im Darmtrakt hin.

P. morganii-Stämme sind im Colon der Säuglinge verbreitet, sie waren ja in 23,6% unserer untersuchten Fälle, also fast aus jeder vierten Stuhlprobe zu isolieren. Bakterienausscheidung, welche von der Jahreszeit unabhängig zu sein scheint, ist in erster Linie bei Säuglingen nachweisbar, was in den Altersgruppen über 1 Jahr mit der Zunahme des Lebensalters allmählich seltener wird. Die Bakterienausscheidung geht nicht mit den enteralen Symptomen parallel einher; manchmal ist sie bereits vor dem Auftreten der Symptome, oder in einer der Phasen der Krankheitsverlaufs, ein anderes Mal erst in der Rekonvaleszenz zu beobachten. Nicht selten lassen sich aus den Stuhlproben des gleichen Kranken in verschiedenen Zeitpunkten immer andere

Serogruppen isolieren. *P. morganii* Stammgruppen sind während der Antibiotikatherapie, in vielen Fällen im Anschluss daran, auch nach Schwinden der klinischen Symptome im Stuhl oft aufzufinden. Diese Beobachtungen dürften mit der Resistenz der Stämme gegen Antibiotika im Zusammenhang stehen und gleichzeitig auch ihre Verbreitung und ihren Fortdauer im Darmtrakt erklären.

Trotz ihrer innerhalb der Krankenhausabteilung zweifellos nachweisbaren Kontakt-Verbreitung, ist eine epidemieartig auftretende »Haus«-Dyspepsie nicht auf *P. morganii* zurückzuführen.

Nach unseren Beobachtungen scheint die *P. morganii*-Gruppe einständiger Vertreter der Darmflora der Säuglinge zu sein, sie lässt sich mit den Gastroenteritiden des Säuglings- und Kindesalters unbekannter Herkunft in keinem ursächlichen Zusammenhang bringen, ihre ätiologische Rolle ist deshalb in diesen Krankheitsformen auf Grund unserer vorliegenden Untersuchungen auch nicht als wahrscheinlich anzusprechen.

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BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

V. UNTERSUCHUNG DER ROLLE DER PSEUDOMONAS AERUGINOSA- UND STAPHYLOCOCCUS AUREUS-STÄMME

Von

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Zusammenfassung. 1. Es wurden Untersuchungen in bezug auf die enteropathogene Rolle der von 376 an enteraler bzw. 638 an extraenteraler Erkrankung leidenden Säuglingen und Kindern stammenden 33 *Ps. aeruginosa* und 25 *Staphylococcus*-Stämmen berichtet.

2. Aus dem Stuhl der enteral Erkrankten liessen sich beide Bakteriengruppen etwas häufiger isolieren, als aus den Stuhlproben der enteral symptomfreien Kranken.

3. Im Stuhl kamen die Serogruppen O:4 und O:5 der *Ps. aeruginosa* relativ häufiger vor. Von den *Staphylococcus*-Stämmen liessen sich diejenigen, die aus extraenteralen Krankheitsprozessen stammen, in die Serogruppe 4, die aus Stuhlproben stammenden Stämme meist in die Serogruppe 2 einteilen.

4. Bezüglich Antibiotika-Empfindlichkeit zeichneten sich die aus Stuhlproben stammenden *Staphylococcus*-Stämme — namentlich die in die Serogruppe 2 einreihbaren Stammtypen — durch ihre Polyresistenz, die aus extraenteralen pyogenen Krankheitsprozessen isolierten, meist in die Serogruppe 4 gehörenden Stämme durch ihre Polysensitivität aus.

5. Die den *Ps. aeruginosa*-Stämmen zuzuschreibenden Darmkatarrhe sind im Säuglingsalter, diejenigen von *Staphylococcus*-Herkunft im Säuglings- und Kindesalter gleicherweise anzutreffen.

6. Unter dem Einfluss einer gezielten Antibiotikatherapie ist entschiedene klinische RepARATION und das Aufhören der Bakterienausscheidung zu beobachten.

7. Die individuelle Untersuchung der enteralen Kranken auf Grund der Analyse der klinischen und Laboratoriumsangaben macht die enteropathogene Rolle der *Ps. aeruginosa*- und *Staphylococcus*-Stämme in den Gastroenterocolitiden des Säuglings- und Kindesalters wahrscheinlich.

(A) PSEUDOMONAS AERUGINOSA

Ps. aeruginosa ist als Erreger verschiedener extraenteraler Infektionen seit langem bekannt. Ihre enteropathogene Rolle ist noch wenig geklärt, doch nimmt man allgemein an, dass sie für manche Enterocolitiden in Säuglings- und Kleinkindesalter verantwortlich gemacht werden kann. Nach EGGERS und WÖCKEL [1] verursachte dieser Keim in einer Kinderklinik eine Enteritisepidemie. HUNTER und ENSIGN [2] beobachteten eine durch *Ps. aeruginosa* ausgelöste Enteritisepidemie wobei Milch die Infektionsquelle war.

Mit der Gruppierung der *Ps. aeruginosa*-Stämme befassten sich zahlreiche Autoren. In neuester Zeit beschrieben HABS [3], KÖHLER [4, 5], KLEINMAIER [6], KLEINMAIER und MÜLLER [7], weiters KLEINMAIER und

QUINCKE [8] Methoden zur serologischen Bestimmung und berichteten über die Häufigkeit der einzelnen serologischen Gruppen in verschiedenem Untersuchungsmaterial (Urin, Eiter, Galle, Stuhl usw.). Die einzelnen Serogruppen zeigten je nach Untersuchungsmaterial eine gewisse charakteristische Verteilung; in bezug auf ihre enteropathogene Rolle gaben jedoch obige Arbeiten keinen Aufschluss.

Material und Methoden

Die Aufarbeitung des Untersuchungsmaterials und das Isolieren der Stämme wurde mit Hilfe der in den Mitteilungen I–IV dieser Serie angegebenen Methoden durchgeführt. Von den biochemischen Reaktionen prüften wir Pyocyandinbildung im flüssigen Pepton-Glyzerin-Nährboden mit Chloroformausschütteln nach sieben Tagen Inkubation, die Fluorescein-Pigmentbildung mit der von GEORGIA und POE [9] beschriebenen Methode und auf Löfflerschem Serum-Nährboden. Kohlenhydrat- und Alkoholabbau wurde mit der Methoden von SIMON [10] geprüft.

Unsere Stämme konnten wir unter Anwendung der aus den früher, sowie im Laufe der vorliegenden Arbeit isolierten Pyocyandin bildenden Stämmen gewonnenen Seren in sieben Antigengruppen einteilen. Die Seren wurden von $2\frac{1}{2}$ Stunden lang bei 100 °C gehaltenen Kulturen gewonnen; die Einstellung der Röhrchenagglutination haben wir mit den Antigenen, die ebenfalls durch die $2\frac{1}{2}$ stündige Wärmebehandlung bei 100 °C gewonnen wurden, vorgenommen. Alle Stämme, bei denen der Titer der Kreuzagglutination nicht genügend hoch war, betrachteten wir als zu verschiedenen Gruppen gehörend. Die im Laufe der Untersuchungen isolierten Stämme haben wir nur dann in die einzelnen Gruppen eingeteilt, wenn diese mit den entsprechenden Seren bis zum Titer oder annähernd dazu agglutinierten.

Ergebnisse

Tabelle I wiedergibt das Vorkommen der *Ps. aeruginosa*-Gruppen im Stuhl der untersuchten 1014 Kranken. *Ps. aeruginosa* kam im Stuhl der 376 enteritischen Kranken in 4,7%, im Stuhl der 638 extraenteralen Kranken in 2,3% vor. Von den isolierten 33 Stämmen konnten wir 28 in fünf Serogruppen verteilen. Auf Grund der auf dem Löfflerschen Nährboden erfolgten braunen oder grünen Pigmentbildung bzw. des Abbaues der Kohlenhydrate und Alkohole konnten wir innerhalb der Serogruppen Biotypen separieren.

Stämme, die Antigene O:1 und O:2 enthielten, konnten aus Stuhlproben nicht isoliert werden. Ein relativ häufigeres Vorkommen der Stämme O:4 und O:5 war bei den an Enteritis leidenden Kranken zu beobachten. Mit Rücksicht auf die geringe Zahl unserer Kranken sind jedoch die im Laufe der gegenwärtigen Untersuchungen gesammelten Angaben bezüglich der Verteilung der Serogruppen und der Beurteilung der enteropathogenen Rolle der *Ps. aeruginosa*-Stämme nicht zureichend.

Ein bedeutender Unterschied zwischen der Antibiotikaempfindlichkeit der vom Stuhl der enteralen Kranken isolierten 18 und von demjenigen der extraenteralen Kranken isolierten 15 Stämmen ist nicht nachweisbar. Gegenüber Polymyxin B fanden wir sämtliche Stämme empfindlich, gegen Penicillin und Erythromycin dagegen alle Stämme resistent. Gegenüber

Tetracycline erwiesen sich vier Stämme als mässig empfindlich, während die übrigen resistent waren. Gegen Streptomycin waren sechs Stämme empfindlich, 15 mässig empfindlich, 12 resistent. Die mässige Empfindlichkeit und Resistenz aufweisenden Stämme gehörten überwiegend in die Serogruppen O:4 und O:5. Gegenüber Chloramphenicol waren mit Ausnahme von zwei mässig empfindlichen Stämmen alle übrigen resistent. 26 Stämme waren neomycinempfindlich, die 7 mässig empfindlichen Stämme gehörten ohne Ausnahme der Serogruppe 4 an.

Tabelle I

Serologische Gruppenverteilung der isolierten 33 *Pseudomonas aeruginosa*-Stämme

Antigengruppe »O«	Enterale Kranken (Stuhl)	Extraenterale Kranken (Stuhl)	Insgesamt
Zahl der unter- suchten Kran- ken	376	638	1014
3	3	2	5
4	7	4	11
5	6	2	8
6	—	3	3
7	—	1	1
ND*	2	3	5
Insgesamt	18	15	33

*ND = in die Gruppen 1—7 nicht einreihbare Stämme

Klinische Beobachtungen. Wie bereits erwähnt konnten wir aus dem Stuhl von 33 Spitalkranken *Ps. aeruginosa* isolieren; 18 dieser Kranken hatten enterale Symptome, 15 waren enteral symptomfreie Bakterienausscheider. Von den 18 Kranken litten zwei an schwerer, neun an mittelschwerer, sieben an leichter Gastroenteritis bzw. Enterocolitis. Die Symptome waren leichte Zyanose, Temperaturerhöhung bis 38—39 °C mitunter auch 40 °C, Erbrechen ein- bis zweimal, in schwereren Fällen mehrmals täglich, 6—10 wässrige, schleimige Stühle. Die häufig grünliche Farbe und der typische Geruch der Stühle liessen allein schon auf das Vorhandensein von *Ps. aeruginosa* schliessen. *Ps. aeruginosa* Massenkulturen erschienen auch auf nicht selektivem Nährboden (Blutagarplatte).

Die enteralen Symptome liefen — unter Anwendung entsprechender Antibiotika (Neomycin, Polymyxin B, Streptomycin) und auf die symptomatische Therapie — in der Mehrzahl der Fälle in 6—12 Tagen ab. Bei 3 Kranken konnten wir drei Wochen lang grünliche, schleimige Stühle und

Bakterienausscheidung intermittierenden Charakters beobachten. Ein Säugling wurde infolge einer 7 Tage nach der Geburt erworbenen, seit 2 Monaten bestehenden chronischen Enterocolitis und konsekutiven Atrophie in Behandlung genommen. Bei 6 Kranken traten als Komplikationen Otitis, Mastoiditis bzw. Bronchopneumonie auf. Die enteral nicht betroffenen 15 Bakterienausscheider litten ebenfalls grösstenteils an Oto-Mastoiditis. In 4 Fällen

Tabelle II

Altersverteilung der untersuchten 33 Kranken

	1—3 Monate alt	4—6 Monate alt	7—9 Monate alt	10—12 Monate alt	Über ein Jahr	Insgesamt
Enterale Kranken	10	3	3	2	—	18
Extraenterale Kranken	4	3	1	3	4	15

liessen sich aus dem bei der Operation gewonnenen Eiter des Mittelohres bzw. des Antrums *Ps. aeruginosa*-Stämme züchten, deren Antigengruppe mit dem Fäkalienstamm des betreffenden Säuglings identisch war. Nach der in klinischem Sinne genommenen Genesung der enteralen Kranken, hörte ihre Bakte-

Tabelle III

Monatliche Verteilung der aus Stuhl isolierten 33 *Ps. aeruginosa*-Stämme

	Insgesamt	Monate											
		I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.	XII.
Enterale Kranken	18	1	—	2	6	2	—	—	—	3	3	1	—
Extraenterale Kranken . .	15	3	2	2	4	—	—	—	1	1	1	—	1

rienausscheidung entweder gänzlich auf, oder war diese nur bis zum 7. Tag der Beobachtungszeit wahrnehmbar. Das Verhältnis der in der Rekonvaleszenz züchtbaren *Ps. aeruginosa*-Kolonien bezogen auf die übrigen Glieder der Darmflora ist mit dem dyspeptischen Stadium verglichen schon äusserst minimal. Kein Patient wurde verloren.

In Hinsicht auf die Altersverteilung waren die enteralen Kranken Säuglinge, meist im I. Trimenon (Tabelle II). Die Gruppe der extraenteralen Kranken bildenden symptomfreien Keimträger sind sowohl in der Altersgruppe unter einem Jahr als auch in derjenigen über ein Jahr aufzufinden. Infolge des postnatalen immunbiologischen Zustandes der Säuglinge ist es nicht zu wundern, das auch von den *Ps. aeruginosa*-Stämmen zuzuschrei-

benden Darminfektionen — den Colidyspepsien analog — in erster Linie die Säuglinge in I—II. Trimenon betroffen werden.

Bei Untersuchung des saisonalen Vorkommens der *Ps. aeruginosa* ist festzustellen, dass die Mehrzahl der Stämme in den Frühjahrs- und Herbstmonaten zur Isolierung kommt (Tabelle III). Das Auftauchen der dominierenden Stämme mit dem Antigenzeichen O:4 und O:5 fällt ebenfalls auf diese Perioden.

Unsere Fälle — abgesehen von 3 nosokomialen Infektionen — ergaben sich aus sporadischen Gastroenterocolitiden. Unsere sich im Gange befindlichen Untersuchungen eines grösseren klinisches Materials, haben den Zweck, zahlreiche Probleme in Verbindung mit der *Ps. aeruginosa*-Gruppe (Ätiologie, immunologische Beziehungen, Epidemiologie) ausführlicher zu behandeln und über dieselben Aufschluss zu geben.

(B) STAPHYLOCOCCUS AUREUS

Bei den Dyspepsien des Säuglingsalters unbekannter Herkunft wird dem *Staphylococcus aureus* eine Rolle zugeschrieben. Nach der Literatur kann *Staphylococcus aureus* von leichter Dyspepsie bis zum »choleriformen Syndrom« [11] oder bis zur chronischen Enteritis, Krankheitsformen verschiedener Schwere verursachen. Zu gleicher Zeit ist der Keim auch aus dem Stuhl gesunder Säuglinge nachweisbar. CISTIVIC [12] isolierte *Staphylococcus aureus* bei 26% der Kinder unter 3 Jahren GRÜN [13] fand den Keim in Stuhl von 31% der Erwachsenen, PRISSICK [14] bei 45,5% von Säuglingen mit Durchfall und bei 46% der Kontrollen. Nach unseren früheren Beobachtungen [15] lässt sich unter den sich im Krankenhaus gepflegten Säuglingen — wegen der günstigen Infektionsbedingungen — *Staphylococcus aureus* in höherem Prozentsatz nachweisen. Trotz der vielen Angaben in der Literatur ist die enteropathogene Rolle der *Staphylococcus aureus*-Gruppe, namentlich in Beziehung zu den Dyspepsien des Säuglingsalters noch nicht ganz geklärt. Zu überzeugenden Beweisen führten bloss die in Verbindung mit gehäuft vorgekommenen alimentären Intoxikationen gewonnenen Beobachtungen.

Material und Methoden

Wir bestimmten die Eigenschaften der von den untersuchten 376 enteralen Kranken aus dem Stuhl von 11, von den 638 enteral symptomfreien Kranken aus dem Stuhl von 14 Kranken, ferner aus dem gemischten Sekret von 24 an extraenteraler pyogener Infektion leidenden Säuglingen und Kinder stammenden 49 *Staphylococcus aureus*-Stämme und arbeiteten die klinischen Angaben der Kranken auf.

Zur Züchtung der Stämme verwendeten wir Blutagarplatten. Wir untersuchten die Koloniemorphologie der sich für *Staphylococcus aureus* erweisenden Kulturen und auf Grund des mikroskopischen Bildes der gefärbten Objektträger-Präparate. Zwecks Identifizierung

prüften wir Nitratreduktion, Ammoniakutilisation, Pigmentbildung, Mannitspaltung und Gelatineverflüssigung nach den durch BERGEY angegebenen Methoden.

Auf die pathogenen Eigenschaften folgerten wir auf Grund der Hämolyisin- (Schaffblutagar), Coagulase- [17], Phosphatase- [18] und Hyaluronidase- [19] Bildung.

Die antigenstrukturelle Analyse nahmen wir auf Grund des von OEDING [20] angegebenen Nachweises der Faktorantigene mittels Objektträger-Agglutination in durch Erschöpfung gewonnenen Faktorseren vor [21]. In Kenntnis der Faktorantigene führten wir die Serogruppierung der Stämme nach OEDING und WILLIAMS [22] durch.

Die Antibiotika-Empfindlichkeit der Stämme bestimmten wir in der in unseren vorherigen Mitteilungen beschriebenen Weise.

Ergebnisse

Die untersuchten 49 *Staphylococcus aureus*-Stämme bildeten Hämolyisin, Hyaluronidase und Koagulase ohne Rücksicht auf ihre Herkunft. Auch die Phosphataseaktivität war — mit Ausnahme von vier Stämmen — bei sämtlichen Stämmen nachweisbar. In Kenntnis dieser Eigenschaften konnte die

Tabelle IV

Serologische Gruppenverteilung der isolierten 49 *Staphylococcus aureus*-Stämme

Serogruppe	Extraenterale Kranken (Eiter usw.)	Enterale Kranken (Stuhl)	Extraenterale Kranken (Stuhl)	Insgesamt
1	1	1	1	3
2	6	3	7	16
3	4	2	—	6
4	12	2	4	18
ND*	1	3	2	6
Insgesamt	24	11	14	49

* ND = Stämme, die in die Serogruppen 1—4 nicht eingereiht werden können

potentielle Pathogenität unserer isolierten Stämme angenommen werden. Von den untersuchten 49 Stämmen stammten 24 aus dem pyogenen Sekret extraenteraler Krankheitsprozesse, 25 aus Stuhlproben. Die Verteilung der *Staphylococcus aureus*-Stämme je nach Serogruppen in den pyogenen Vorgängen der extraenteralen Kranken ferner im Stuhl der enteral kranken bzw. symptomfreien Säuglinge und Kinder ist in Tabelle IV angeführt.

Die Gruppeneinteilung von OEDING und WILLIAMS [22] folgend, sind 43 unserer Stämme in die Serogruppen 1—4 einzureihen, während sechs Stämme nicht klassifiziert werden konnten. Aus den Angaben der Tabelle IV geht hervor, dass 50% der aus den extraenteralen pyogenen Infektionen isolierten Stämme in die 4. Serogruppe eingereiht werden kann. Die Fäkalstämme der an Durchfall leidenden Kranken zeigen keine Polarisation. Demgegenüber

sind im Stuhl der enteral symptomfreien Ausscheider die in die 2. Serogruppe gehörenden Stämme verhältnismässig häufiger. Unsere Befunde in bezug auf die Gruppenverteilung der *Staphylococcus aureus*-Stämme dürften jedoch nur durch ausgedehnte, sich auf ein grösseres — innerhalb und ausserhalb des Krankenhauses erworbene Fälle umfassendes — Krankenmaterial erstreckende Untersuchungen geklärt werden.

Bei den Antibiotika-Resistenzprüfungen *in vitro* haben wir keinen sulphonamidempfindlichen, oder gegen Neomycin und Bacitracin resistenten Stamm gefunden. Penicillinempfindlichkeit war nur bei (14,3%) der Stämme

Tabelle V

In vitro Resistenz der isolierten 40 *Staphylococcus aureus*-Stämme

Antibiotikum	Empfindlich		Resistent	
	Zahl	%	Zahl	%
Sulfonamid	0	0,0	49	100,0
Penicillin	7	14,3	42	85,7
Streptomycin	25	51,0	24	49,0
Chloramphenicol	28	57,1	21	42,9
Chlortetracyclin	26	53,0	23	47,0
Oxytetracyclin	22	44,9	27	55,1
Neomycin	49	100,0	0	0,0
Erythromycin	47	95,9	2	4,1
Bacitracin	49	100,0	0	0,0

nachzuweisen. Gegenüber Streptomycin bzw. Tetracycline erwiesen sich 45—55% der Stämme als empfindlich. Eine ähnliche Empfindlichkeit wie gegenüber Neomycin zeigte 96% der Stämme auch gegenüber Erythromycin. Die Ergebnisse der Resistenzprüfungen wurden in Tabelle V zusammengestellt.

Wir möchten betonen, dass sich diese Angaben auf unser Spitals-Krankengut vom Jahre 1958 beziehen. Seitdem engte sich nämlich die Antibiotikaempfindlichkeit der *Staphylococcus aureus*-Stämme — namentlich die der Fäkalstämme — *in vitro*, aber auch *in vivo*, entschieden ein.

Die isolierten 49 Stämme lassen sich — nach ihrem Antibiotogramm — in polysensitive und polyresistente Typen teilen. (Polysensitiv: gegen zwei Antibiotika resistent, gegen die übrigen empfindlich; polyresistent: gegen ein bis drei Antibiotika empfindlich, oder mässig empfindlich, gegen den übrigen resistent.) Nach unseren Beobachtungen ist ein Zusammenhang zwischen der Resistenz und der Antigengruppe der Stämme nachzuweisen. Die polysensitiven Stämme gehörten grösstenteils in die 4., die polyresistenten dagegen in die 2. Serogruppe (Tabelle VI).

Es kann weiterhin festgestellt werden, dass die Mehrzahl der polysensitiven Stämme der 4. Serogruppe aus pyogenen Krankheitsprozessen stammen, während die in die 2. Serogruppe gehörenden polyresistenten Stammtypen aus den Stuhlproben von enteral-kranken und symptomfreien Säuglingen herrühren.

Klinische Beobachtungen. Von den 49 *Staphylococcus aureus* positiven Fällen beanspruchen in erster Linie diejenigen das Interesse, bei denen enterale Symptome im Vordergrund standen (11 Kranken). Die Angaben und die

Tabelle VI

Zusammenhänge zwischen den Serogruppen
und der Antibiotikaempfindlichkeit der *Staphylococcus*-Stämme

	Serogruppen				ND*	Insgesamt
	1	2	3	4		
Polysensitiv	2	4	3	15	2	26
Polyresistent	1	12	3	3	4	23
Insgesamt	3	16	6	18	6	49

* ND = in Serogruppen 1—4 nicht einreihbare Stämme

Staphylococcus aureus-Stämme der 14 symptomlosen Bakterienausscheider bzw. der 24 an extraenteraler pyogener Infektion (Empyem, Oto-Mastoiditis, Pyodermen) Leidenden dienten bloss zum Vergleich. *Staphylococcus aureus*-Stämme liessen sich in 2,9% des untersuchten enteralen Materials (376 Fälle) und in 2,2% des Kontrollmaterials (638 Fälle) nachweisen. Die geringe Zahl der isolierbaren *Staphylococcus aureus*-Stämme erklärt der Umstand, dass wir zu ihrer Züchtung nicht die übliche selektive NaCl-Agarplatte angewendet haben. Die Enteritiden von nehmbarer *Staphylococcus aureus*-Herkunft unserer 11 Kranken verdienen vielleicht eben deshalb trotz der geringen Zahl der Fälle grössere Aufmerksamkeit.

Von den 11 Kranken litten einer an schwer, 4 an mittelschwer, 6 an leicht ablaufender klinischer Enteritis. Eine auf alimentäre Intoxikation zurückzuführende Gastroenteritis konnte bloss bei einem Kranken beobachtet werden. Charakteristische Symptome waren 5—6mal täglich entleerte Stühle dünner, wässriger Konsistenz, 1—2 Erbrechen, 38—39 °C Fieber und Mattigkeit. Die klinischen Symptome liefen — unter der gezielten antibiotischen Behandlung — in 7—10 Tagen ab. Dem richtig gewählten Antibiotikum bzw. ihren Kombinationen ist die frappante Ausheilung des Darmprozesses und das Fehlen extraenteraler Komplikationen zuzuschreiben. Besonders im Anfangsstadium der Krankheit war aus den vor dem Beginn der Antibiotikatherapie gewonnenen Stuhlproben auf Blutagarplatte *Staphylococcus aureus* in Rein-

kultur isolierbar. 3—4 Tage nach Aufhören der enteralen Symptome war *Staphylococcus aureus* aus dem Stuhl nicht mehr nachzuweisen.

Innerhalb des Krankenhauses erworbene Enteritis war bei zwei Kranken zu beobachten; es liessen sich aus dem Stuhl beider Säuglinge gleicherweise die in die 2. Serogruppe gehörenden Stämme züchten. Bei weiteren 9 Kranken wiesen die anamnestischen Angaben und der *Staphylococcus aureus*-Befund der im Anschluss an die Aufnahme ins Krankenhaus durchgeführten bakteriologischen Stuhluntersuchung auf das sporadische Vorkommen der Fälle hin. Die Anwesenheit bekannter enteropathogener Keime konnte auch bei wiederholten Stuhluntersuchungen nicht nachgewiesen werden. Hinsichtlich der Verteilung der 11 enteritischen Kranken je nach Alter waren 6 Säuglinge und 5 Kleinkinder über ein Jahr.

Besprechung

Unsere Bestrebung, eine Aufklärung während der einjährigen Beobachtungszeit über die enteropathogene Rolle der *Ps. aeruginosa*- und *Staphylococcus aureus*-Stämme bei den Darminfektionen des Säuglings- und Kindesalters und in bezug auf die serologische Verteilung der Stämme zu erhalten, führte infolge der geringen Zahl der Fälle zu keinen statistisch verwertbaren Schlussfolgerungen. Dennoch konnten die gesammelten Angaben neben Klärung der Fragen in bezug auf die Rolle der sonstigen untersuchten Darmbakterien, innerhalb der gegebenen Zeitperiode über die ursächliche Rolle der *Pseudomonas* und *Staphylococcus*-Stämme gewissermassen Aufschluss geben.

In unserem Untersuchungsmaterial ist das Vorkommen dieser beiden Bakteriengruppen nicht bedeutend (4—5%) und auch im Verhältnis zu den Angaben in der Literatur ausserordentlich gering. Die angewendeten Züchtungsmethoden, das sporadische Vorkommen der enteralen Fälle und die geringe Zahl der in der Krankenhaus-Abteilung vorkommenden *Ps. aeruginosa*- und *Staphylococcus*-Ausscheider dürften unsere von den Mitteilungen in der Literatur abweichenden Beobachtungen erklären. Die Häufung der nosokomialen Infektionen ist nämlich in der Regel die Funktion der Zahl der Abteilungs-Bakterienausscheider, wie dies auch unsere neueren Untersuchungen nachweisen. Vom Gesichtspunkt der Ätiologie aus kann auch unserer Beobachtung eine Bedeutung beigemessen werden, wonach die Auffindbarkeit dieser beiden Bakteriengruppen im Stuhl der enteralen Kranken im Verhältnis zu den Bakterienausscheidern nahezu zweimal so häufig ist. Von den den *Pseudomonas*- und *Staphylococcus*-Stämmen zuzuschreibenden Dyspepsien werden in erster Linie — ähnlich wie bei anderen Darmbakteriengruppen — Säuglinge betroffen, obwohl *Staphylococcus*-Enteritiden von auch in den Altersgruppen über

ein Jahr diagnostiziert werden können. Auffallend ist die hochgradige Antibiotikateranz der aus dem Stuhl isolierten Stämme, die auf diese Weise infolge ihrer Verbreitung im Wege der natürlichen Stammselektion und durch den Stuhl ein Kettenglied des epidemiologischen Problems der nosokomialen *Pseudomonas* und *Staphylococcus*-Infektionen bilden dürften. Von Neomycin, das gegen beide Bakteriengruppen *in vitro*, aber auch *in vivo* gleich wirksam ist, sind heute noch günstige therapeutische Ergebnisse zu erwarten.

Die individuell vorgenommene Untersuchung der an enteralen Symptomen leidenden Säuglinge bzw. Kinder unterstützen, neben der Ausschliessbarkeit der bekannten Enteropathogene, auf Grund der Analyse der klinischen und Laboratoriumsangaben die bei den Darminfektionen gespielte, auch von anderen aufgeworfene kausale Rolle der *Ps. aeruginosa* und *Staphylococcus*-Stämme. Die geringe Zahl der anlässlich der gegenwärtigen Untersuchungen gesammelten Fälle beeinflusst die Verwertbarkeit unserer Beobachtungen in hohem Masse. Die ständig wachsende Zahl der *Staphylococcus* und *Ps. aeruginosa*-Krankheiten in den Spitalstatistiken veranlasst uns, diesen Problemkreis auf breiterer Basis aufzuarbeiten.

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DOMINANCE IN HYBRIDS DERIVED FROM CROSSING OF STREPTOMYCETES

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Summary. *Streptomyces rimosus* has been crossed with a non-streptomycin-producing *S. griseus* strain, and with a broad spectrum antibiotic-producing *S. globisporus* strain. In regard to the chosen markers (shape or existence of aerial mycelium, shape and colour of colonies, gelatin liquefaction, antibiotic spectrum or nature of antibiotic), the dominance of the most decisive characters of one of the parents has been revealed in the hybrids. The characters of *S. griseus* and *globisporus* were dominant, those of *S. rimosus* were recessive. The production and shape of the aerial mycelium, gelatin liquefaction and the nature of antibiotic were dominant properties. The shape of colonies and the amount of the produced antibiotics were not dominant features and differed from the corresponding parental properties. Antibiotic production was hardly detectable in hybrids derived from highly antibiotic producer parents. Dominance as to the chosen markers was observed only when dominant and recessive species were mated. Crossing of recessive species resulted in highly variable hybrids.

Several papers have been published on nuclear fusion and recombination following the anastomosis of streptomycetes [1—9]. It has been shown that among the isolates derived from the nuclear fusion following anastomosis of two species, several segregants may be found, which resemble neither of the parents [8]. The problem whether the characters of one of the mating partners dominate in the isolates has not been investigated, but experience has shown that in certain cases the domination very probably exists. Based on this conception, we divided *Streptomyces* strains isolated from soil into two groups *viz.* (i) organisms with stable hereditary properties; and (ii) organisms with unstable hereditary properties [10]. In former studies the presence of stable or unstable characters was attributed to the properties of the soil. It has been thought, for example, that stable species occur in rapidly drying small sodic spots, because only quickly sporulating organisms are able to survive under such conditions. Alternatively, the unstable species live in soils which are permanently wet throughout the year. In our present opinion, that concept is of limited value. Heterokaryosis may undoubtedly be produced as a result of environmental conditions (permanently wet soil). The stability and instability of organisms is, however, an entirely different problem. Some characters of unstable isolates were described previously [8]. The stable hybrids are dealt with in the present paper.

Materials and methods

After long lasting preliminary experiments two *Streptomyces* species with apparently non-variable, stable properties were chosen. One of the strains was a non-antibiotic-producing variant of *S. griseus*, isolated and designated as strain 14 at the *Department of Microbiology, Institute of Agricultural Research, Polany*. The other strain, *S. globisporus*, was isolated in this laboratory from a sodic soil sample obtained in the *Hungarian Great Plain* [11]. *S. rimosus* strain Ja 4696 was considered as a genetically suppressible organism. This strain produced no aerial mycelium on media not enhancing spore formation.

No auxotrophic markers were used, because 6 years' investigations revealed that any auxotrophic clone exerted back-mutation even when 2 markers were considered. It has therefore been concluded that experiments with auxotrophs give unconvincing results. Accordingly, in the present studies morphological and physiological characters were considered as follows. Morphological characters: straight (*S. griseus* and *globisporus*) or spiral (*S. rimosus*) sporophore; colonial morphology, sporulation (*S. griseus* and *globisporus*) lack of aerial mycelium (*S. rimosus*): presence or absence of antibiotic production, and the nature of antibiotic.

As no biochemical dependence markers were used, and there is no proof of recombination having occurred, in this paper the terms hybridization and hybrids are used.

Experimental

The experiments were performed in two parts: (i) *S. griseus* was mated with *S. rimosus*; (ii) *S. globisporus* was mated with *S. rimosus*.

(i) For obtaining a successful mating, *S. griseus* was trained to graded concentrations of oxytetracycline until a 1000 $\mu\text{g/ml}$ resistance had developed. As the original *S. griseus* produced no antibiotic, it was necessary to develop a resistant form capable of growing together with the oxytetracycline-producing *S. rimosus*. Mating was performed on Lindenbein glycerol agar [12]. The two strains were inoculated onto the same medium. After 4 days the mixed culture was transferred to LINDENBEIN'S medium. After subsequent incubation for 7 days, the aerial mycelium and spores were collected with a loop. The material was shaken on a horizontal shaker, in a flask containing distilled water and glass beads. Various dilutions of the suspension were plated onto WAKSMAN'S meat extract-peptone agar [13]. The plates were incubated at 29° C for 7 days. As controls, the parent organisms were also plated onto the above medium. In the cultures colonies of different morphology were observed. Among 30 colonies generally 2 or 3 differed from the others. These were isolated, subcultured and then homogenized by glass bead grinding. Cultures with stable properties were examined further. At first, their stable colonial morphology was confirmed by subcultivation and plating. The stable characters indicated that the isolates were hybrids brought about by nuclear fusion following the anastomosis of two different species. The hybrids were thoroughly examined and compared with the parents as to growth on various media. The behaviour of the cultures on some of these media is presented as follows.

(a) WAKSMAN'S meat extract agar. *S. griseus*: the aerial mycelium is white with a light yellowish shade; *S. rimosus*: yellow substrate mycelium,

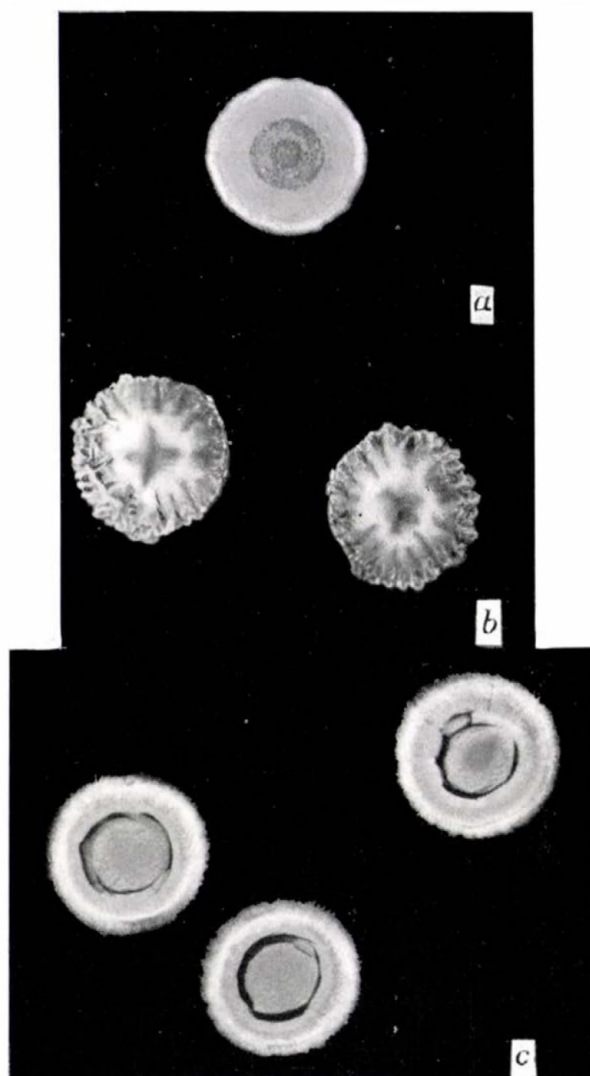


Fig. 1. Colonies on potato agar of (a) *S. griseus*, (b) *S. rimosus*, (c) their hybrids

produces no aerial mycelium; griseus/rimosus (hereinafter *g/r*): light grey aerial mycelium, the reverse side of the colony is light brown.

(b) Potato agar. *S. griseus* : greyish aerial mycelium, the middle of the colony is of a characteristic deeper shade; *S. rimosus* : yellow substrate mycelium, no aerial mycelium; *g/r*: light grey aerial mycelium, colonies are differentiated after one week to one inner and one or two outer parts.

The colonies are shown in Fig. 1.

(c) Potato. *S. griseus*: white aerial mycelium, later the potato turns to brown; *S. rimosus*: produces only brownish substrate mycelium; g/r: aerial mycelium is darker grey than in *S. griseus*.

(d) LINDENBEIN's glycerol agar. *S. griseus*: white, yellowish shaded aerial mycelium, reverse of colony and agar are not coloured; *S. rimosus*: yellowish, light brown substrate mycelium, no aerial mycelium; g/r: strikingly different from *S. griseus* in its greenish-shaded light greyish-white aerial mycelium forming a thick coating over the medium after two weeks.

(e) Gelatin is not liquefied by any of the cultures.

The aerial mycelium is straight in *S. griseus*, spiral in *S. rimosus*, and somewhat curved in g/r.

The antibiotic production by the parents and hybrids was tested by the cross-streak technique (Table I).

It is seen that, as compared to the highly active *S. rimosus*, the *S. griseus* strain produced very small amounts of antibiotic effective against bacteria belonging to Enterobacteriaceae. The same holds true for the four g/r hybrids, the antibiotic patterns of which are almost identical with that of *S. griseus*. The identity should have been confirmed by paper chromatography. However, the antibiotic was produced in such minimum quantities that we were unable to obtain sufficient amounts for the analysis.

(ii) Mating of two broad spectrum antibiotic-producing organisms,

Table I

Antibiotic production by *S. griseus*, *S. rimosus* and hybrid strains

	<i>S.</i> <i>rimosus</i>	<i>S.</i> <i>griseus</i>	g/r 1	g/r 2	g/r 3	g/r 4
<i>Staph. aureus</i>	15*	—	—	—	5	4
<i>B. subtilis</i>	15	5	—	5	7	9
<i>E. coli</i>	10	—	—	—	—	—
<i>B. radiobacter</i>	10	—	—	—	—	—
<i>Sarcina lutea</i>	15	10	—	6	5	5
<i>S. marcescens</i>	—	—	—	—	—	—
<i>Proact. roseus</i>	10	4	—	—	—	—
<i>Str. griseus</i>	10	10	18	17	8	15
<i>P. chrysogenum</i>	10	20	17	20	20	24
<i>M. peregrinum</i>	10	10	15	10	8	8
<i>M. phlei</i>	—	10	15	12	—	—
<i>M. smegmatis</i>	10	10	—	10	—	—

* Figures mean the zone of inhibition in mm.

S. globisporus and *S. rimosus*. *S. globisporus* produced a new antibiotic [14], acting on a variety of Gram positive and negative bacteria [11, 15]. The two species differed in some respects: *S. rimosus* produced no aerial mycelium on organic medium, while *S. globisporus* sporulated well; in contrast to the

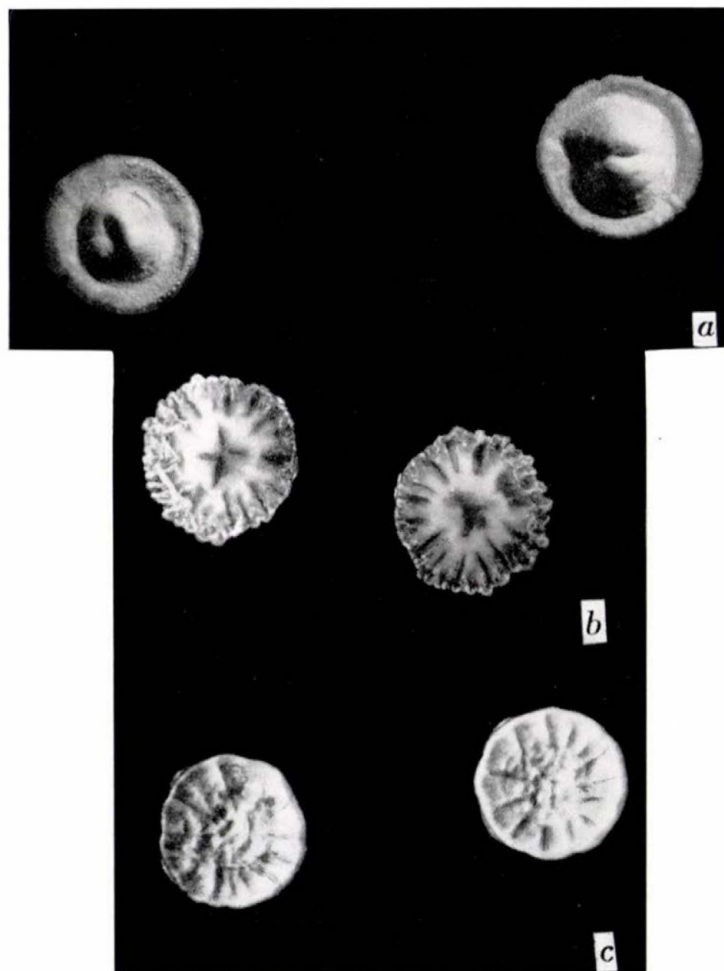


Fig. 2. Colonies on potato agar of (a) *S. globisporus*, (b) *S. rimosus*, (c) their hybrids

spiral aerial mycelium of *S. rimosus*, *S. globisporus* formed straight aerial mycelium.

The mating technique involving the induction of mutual antibiotic resistance failed with these organisms, because *S. globisporus*, although it had been passed through a number of subcultures, could not be trained to grow in the presence of even 10 $\mu\text{g/ml}$ oxytetracycline. On the other hand, in syn-

thetic media *S. globisporus* was highly inhibitory to *S. rimosus*. Finally, mating was successful in the following manner. The spores of *S. globisporus* were sprinkled over the substrate mycelium of a 32 hour agar slant culture of *S. rimosus*. As immediately after spore germination the organism produced no antibiotic, it was able to anastomize with *S. rimosus*. After two weeks, incubation the culture was homogenized in sterile water by glass bead grinding on a horizontal shaking apparatus. The suspension was passed through filter paper and plated onto potato agar. Four kinds of well differentiated colonies were observed: of 50 colonies 11 were *S. rimosus*, 27 were *S. globisporus*,

Table II
Antibiotic production by S. globisporus, S. rimosus an hybrid strains

	<i>S.</i> <i>rimosus</i>	<i>S.</i> <i>globisporus</i>	rim/glob 1	rim/glob 2	rim/glob 3	rim/glob 4
<i>Staph. aureus</i>	15*	10	—	—	—	—
<i>B. subtilis</i>	15	13	2	3	5	3
<i>E. coli</i>	16	13	—	—	—	—
<i>B. radiobacter</i>	30	15	—	—	—	—
<i>Sarcina lutea</i>	20	15	4	6	7	2
<i>S. marcescens</i>	—	5	—	—	—	—
<i>Proact. roseus</i>	10	10	2	6	6	5
<i>Str. griseus</i>	10	5	—	2	2	—
<i>P. chrysogenum</i>	10	15	—	—	—	—
<i>M. peregrinum</i>	10	5	—	—	—	—
<i>M. phlei</i>	—	5	—	—	—	2
<i>M. smegmatis</i>	10	15	3	5	3	5

* Figures mean the zone of inhibition in mm.

and 8 colonies resembling *S. globisporus* were of doubtful character. Four colonies were distinct from each parent strain. The doubtful colonies were isolated and plated after subsequent homogenization by shaking. As the cultures yielded mainly *S. globisporus* and in a small proportion *S. rimosus* colonies, they were considered as heterokaryons. The four distinct colonies were also isolated and plated after subcultivation and homogenization. The perfectly uniform colonial morphology observed at first plating has been maintained now for 1½ years. Thus the strains corresponded to hybrids originating from nuclear fusion.

Morphologically, the hybrids were uniform in producing slightly curved, almost straight aerial mycelium. In this property they resembled mainly *S. globisporus*. Both *S. globisporus* and *rimosus* sporulated well, while the

hybrids formed only fragments. As to enzyme activity, similarly to *S. globisporus*, the hybrids liquefied gelatin.

Fig. 2 shows the hybrid and parent colonies. As it is seen, in contrast to *S. rimosus*, the hybrid produces aerial mycelium and *S. globisporus* sporulates well. The surface of the hybrid colony is different from that of both parents. The aerial mycelium of the hybrid is somewhat darker grey than the creamy grey aerial mycelium of *S. globisporus*. *S. rimosus* produces even darker grey spores, but only on media which promote sporulation.

The antibiotic activity of the hybrid and the two parent strains is presented in Table II. The zones of inhibition obtained by the cross-streak technique are given in mm.

It is striking that, in contrast to the parents, the hybrid is a poor antibiotic producer. The antibiotic production by rim/glob strain 3 could be enhanced in the fermentor so that the harvest was sufficient for paper chromatographic examinations. Of the three phase antibiotic produced by *S. globisporus*, the R_f value of phase 2 factor was identical with that of the substance produced by the hybrid.

Discussion

As to whether the characters of the parents are manifested dominantly in the hybrids, this seems to be possible with certain species. In a previous paper, a high degree of segregation of the hybrids has been reported [8]. In contrast, as shown by the example of the mating of *S. griseus* and *rimosus*, in the present experiments well-characterized properties of only one of the parents emerged. Similarly to *S. griseus*, the hybrids produced straight aerial mycelium and in antibiotic spectrum they also resembled this organism. The example of the crossing of *S. globisporus* with *S. rimosus* is even more convincing. Similarly to *S. globisporus*, the hybrid produces aerial mycelium. There is a close resemblance in the colour of the aerial mycelium as well. Both cultures liquefy gelatin, and although in minimum amounts, the hybrid produces an antibiotic identical with that of *S. globisporus*. Quantitatively, however, the hybrid differs from the parent strains. While these produce high levels of broad activity spectrum substances, the hybrid is a very poor antibiotic producer. Thus it is evident that two different loci are responsible for the production of the two kinds of antibiotic. Consequently, at hybridization the information for antibiotic production originating from two different loci, is suppressed. Some authors [16, 17] found that recombinants might have a higher antibiotic activity than their parents. Such experiments dealt with inbreed crossings, where the information for antibiotic production was situated in identical loci. Accordingly, antibiotic activity became increased in the recombinants. Mating of different species brings about just the opposite result.

Despite the considerable decrease in antibiotic production in the hybrids of actively antibiotic producing species, there is a genetic dominance, because the antibiotic elaborated by the hybrids is identical with the substance produced by the parent regarded as possessing the dominant characters.

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SOME BIOLOGICAL CHARACTERISTICS OF ADENOVIRUS TYPE 8 STRAINS

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Summary. Twelve strains of adenovirus type 8 have been studied in primary human amnion cell cultures. Two of these were laboratory strains, the remaining 10 were freshly isolated from cases of epidemic keratoconjunctivitis. All the strains gave rise to so-called "type-3 degeneration".

Using standard inoculum, the cytopathic changes, like those caused by other adenovirus types, appeared earlier and became more pronounced in old (30 days old or older) cultures than in young (5–10 days old) ones.

The virus yield ranged from 100 to 300 TCID₅₀ per ml medium.

The applied adsorption period varied from 30 minutes to 5 hours. The earliest appearance of both the first signs of degeneration and the complete degeneration of the cultures was observed after five-hour adsorption.

Using the optimum adsorption period and inocula of 1, 2, 4, 8, 20 or 40 TCID₅₀, three zones were distinguishable. In zone I one day was required for a 25 per cent progress in the degeneration of cells, and a reduction of the dose of virus by 50 per cent prolonged by one day only the time necessary for the same degree of degeneration. In zones II and III both these indices rose to 3 or 4 days and 7 days, respectively. The delay in the appearance, and the slow development, of the specific degeneration in the cultures infected with small doses of virus may explain why the isolation of adenovirus type 8 strains is so tiresome and time-consuming.

It is well-known that the type-8 adenovirus strains, the causative agents of epidemic keratoconjunctivitis (EKC), are sharply different in some biological characteristics from other members of the Adenovirus family [1–5, 16]. One of the most pronounced divergencies is that in tissue culture media type-8 strains never attain titres considerably higher than 100–300 TCID₅₀ per ml. Efforts to raise the titre by changing the techniques of cultivation have been unsuccessful [1, 16], though with other adenoviruses a hundred times higher titres are easily achieved.

When studying the aetiology of the EKC epidemic that occurred in Hungary in 1961–1962 we pointed out that the isolation of type-8 strains required a long period of observation or even several blind passages [6]. It might be assumed that the prolonged latency and the low titre might be two manifestations of the same biological characteristic. Thus, it seemed interesting to study the relation of freshly isolated type-8 strains to the host cells as well as the dynamics of the cytopathic (CP) changes. As controls, two laboratory strains were included in the study.

Materials and methods

Virus strains. Twelve strains of adenovirus type 8 were examined. The strains 8/4, 8/8, 8/9, 8/10, 8/11, 8/12 and 8/13 were isolated during the Budapest epidemic in winter 1961–1962 [6]; the 8/7 strain from a sporadic case of EKC in 1963, whereas the strains 8/5 and 8/6 were re-isolated from two accidental laboratory infections [6]. The strain designated 8/3 and 8/14 were kindly supplied by Dr. R. S. DREIZIN (USSR) and Dr. E. JAWETZ (USA), respectively. The latter strain is identical with the Japanese strain "Ijima".

Tissue cultures. Primary human amnion and embryonic kidney cell cultures were prepared and maintained as usual [7, 9, 10]. The medium for amnion cells contained 10 per cent calf serum and 90 per cent Hanks' solution enriched with 0.4 per cent lactalbumin hydrolysate; that for kidney cell cultures consisted of 20 per cent human serum and 80 per cent Hanks' solution with 0.4 per cent lactalbumin hydrolysate. Immediately before inoculation the cultures were washed several times with Hanks' solution. As maintenance solution a medium containing 5 per cent rabbit serum, and 95 per cent Hanks' solution with 0.25 per cent lactalbumin hydrolysate was used throughout.

Results

All the 12 type-8 strains caused an identical morphological change in tissue cultures. The CP changes were identical with those described by us as "type-3 degeneration" in earlier reports [7]. Starting at the edge of the monolayers the cells rounded off, showed some shrinkage and their refraction became sharper. The cells remained connected with one another by cytoplasmic bridges and so formed a network. At a later stage the network broke off and finally, cluster-like islands consisting of 3–10 rounded cells developed. In some cases network formation was less pronounced, and islands of degenerated cells detached from the edges of apparently intact monolayers. In the rounded cells of both the amnion and kidney cell cultures the nuclei were granulated (Fig. 1) and in some nuclei a "central mass" or other patho-

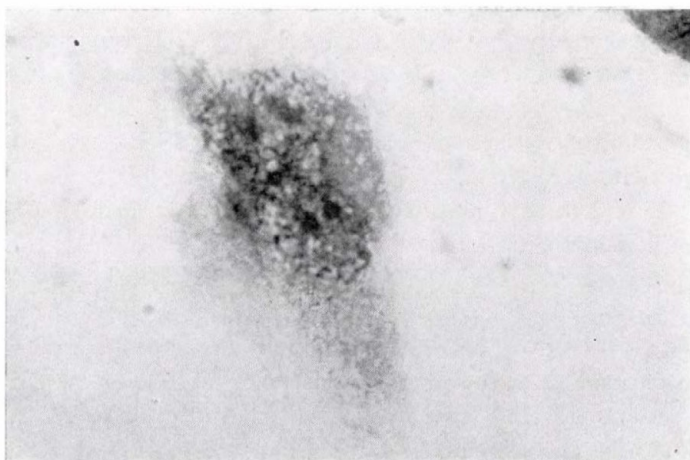


Fig. 1. Cells from human embryonic kidney cell culture two days after inoculation with adenovirus 8/4. Giemsa stain. Magnification, approximately $\times 1200$

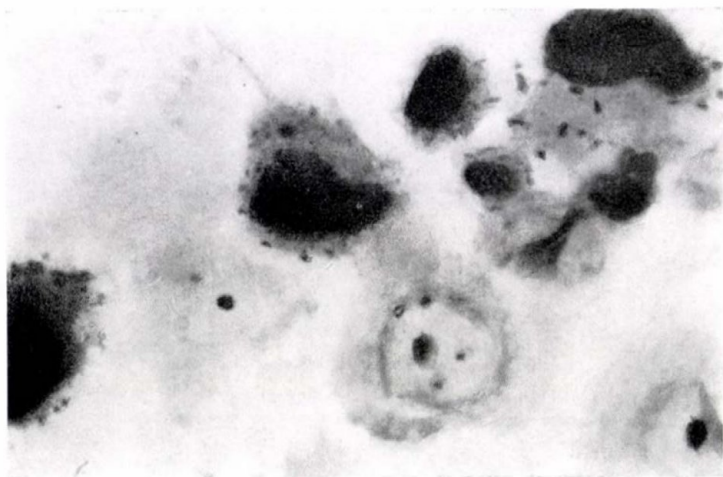


Fig. 2. Human amnion cell culture 5 days after inoculation with adenovirus 8/14 at 7 day of incubation

logical formations were seen [7, 11]. Granules were often observed in the cytoplasm (Fig. 2). Thus the strains under study may be included in the group of the adenoviruses producing "type-3 degeneration".

In the course of an earlier study [10] we demonstrated that the CP effects of the 20 strains of adenoviruses tested were earlier observable in old amnion cell cultures (at least 30-day old when inoculated) than in young ones. It seemed therefore of interest to study whether such differences existed for type-8 adenoviruses, too. Human amnion cell cultures 5 and 48 days of age

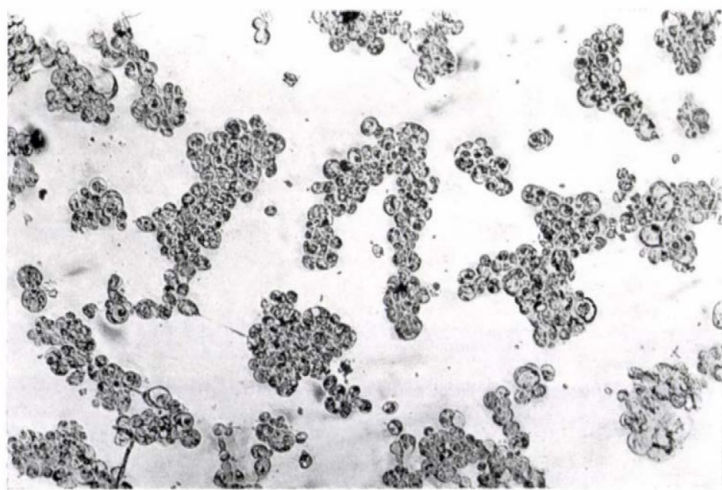


Fig. 3

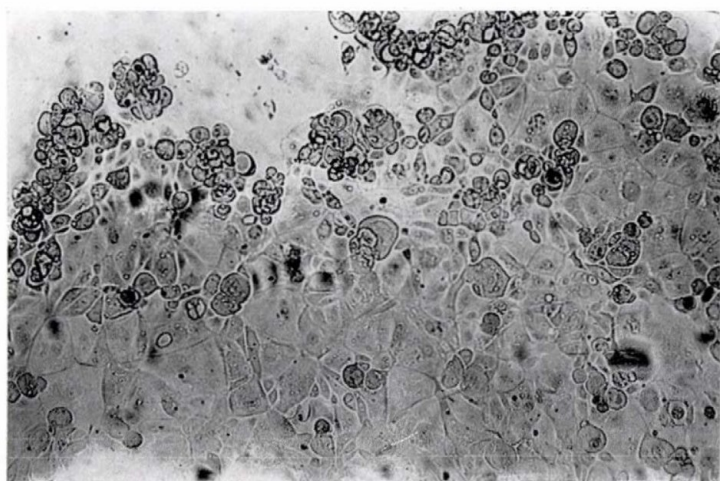


Fig. 4

Figs. 3 and 4. Primary human amnion cell cultures 10 days after inoculation with the same quantity of adenovirus 8/4. The culture in Fig. 3 was 48 day-old, that in Fig. 4 was 5-day-old, when inoculated. Unfixed. Magnification, $\times 130$

were inoculated with 4 TCID₅₀ of the 8/14 strain each. The adsorption time was 90 minutes. The results are illustrated in Fig. 5. Complete degeneration was achieved in both cases, but with a considerable delay in the younger cultures (after 24 days in contrast to 16 days). Figs. 3 and 4 show the degree of degeneration in the "young" and in the "old" cultures 10 days after inoculation. In the "old" culture the degeneration is already complete, nothing

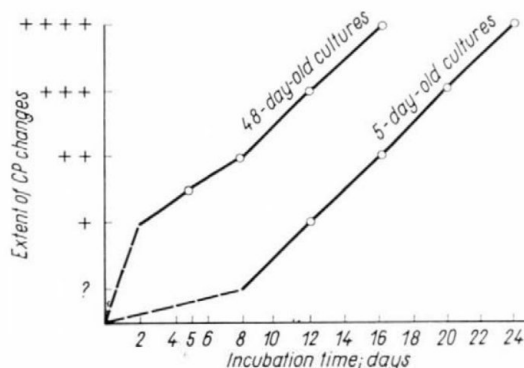


Fig. 5. The progress of degeneration in old and young human amnion cell cultures infected with 4 TCID₅₀ of adenovirus 8/14.

Explanation. ? = initial stage of degeneration, specificity questionable
 + = specific CP foci affecting approx. 25 per cent of the cells
 ++ = 50 per cent of the cells are affected
 +++ = 75 per cent of the cells are affected
 ++++ = total destruction

but degenerative islands are seen, while the "young" culture is affected only at the edge of the monolayer.

In Fig. 6 we wish to visualize the dynamics of the CP changes in old (44 days) and in young (6 days) amnion cultures. In these experiments varying doses of the 8/6 strain were used. The period of adsorption was 90 minutes.

Irrespective of the size of the inoculum, both the appearance of the first CP changes and the complete destruction of the cultures showed a consider-

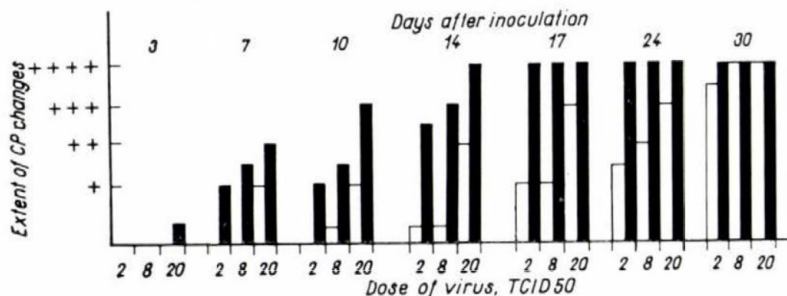


Fig. 6. The progress of CP changes in old and young human amnion cell cultures infected with various doses of adenovirus 8/6

White columns = Cultures 6-day-old when infected

Black columns = Cultures 44-day-old when infected

Further explanation see Fig. 5.

able delay in the young cultures; e.g. by the sixteenth day after inoculation each of the three doses had produced a complete destruction in the old cultures while in the young cultures as much as 30 days did not suffice for the smallest dose to cause total destruction. Similar differences were observed when other type-8 strains were employed showing that in this respect the freshly-isolated and the laboratory strains of adenovirus type 8 behave like the other adenovirus types.

Numerous maintenance fluids were titrated in amnion cell cultures. The results agree with JAWETZ and HANNA's [1, 16] observations on type-8 strains including the Ijima (= 8/14) strain. As a rule, the virus concentration ranged from 100 to 300 TCID₅₀ per ml, except in two cases where the titre attained 400 TCID₅₀ and another case where 700 TCID₅₀ were found in each ml. These high titres were achieved with freshly-isolated strains. We have never observed as high titres as reported by CLYDE and DENNY [17].

In further experiments the adsorption period was varied. Tube cultures of amnion cells were prepared using the initial cell count 200,000 per ml. Various dilutions of the maintenance solution of an amnion culture containing 400 TCID₅₀ per ml were used as inocula; these contained 40, 20, 8, 4, 2 or 1 TCID₅₀ of adenovirus type-8 in 0.1 ml. The cultures inoculated with the same

Table I

Influence of adsorption period on incubation time required for total destruction of human amnion cell cultures infected with 20 TCID₅₀ of adenovirus 8/6

Adsorption, minutes	Incubation, days						
	3	7	10	14	17	24	30
30	—	—	—	+	+	+	++
60	—	—	+	+	+	++	+++
90	—	+	+	+	++	+++	++++
150	—	+	+	++	+++	++++	++++
300	+	++	+++	+++	++++	++++	++++

See footnote to Fig. 5.

Table II

Influence of adsorption period on incubation time required for total destruction of human amnion cell cultures infected with 4 TCID₅₀ of adenovirus 8/4

Adsorption, minutes	Incubation, days							
	2	6	9	12	16	20	23	30
30	—	—	—	—	—	?	?	+
60	—	—	—	—	?	+	+	++
90	—	—	—	—	?	+	+	++
150	—	—	?	?	+	+	++	+++
300	—	?	+	+	++	++	+++	++++

See footnote to Fig. 5

dose were divided into five groups. These were incubated with the inoculum for 30, 60, 90, 150 and 300 minutes. Then the cultures were washed three times with Hanks' solution and further incubated with 1 ml maintenance fluid. The cultures were observed every day. In the experiments shown in Tables I and II, 20 and 4 TCID₅₀ were used, respectively. In both cases as well as after administering the other three doses, adsorption of 300 minutes was followed by the earliest appearance of both the first CP changes and the complete destruction.

Fig. 7 visualizes the dynamics of cell degeneration as the function of the size of the inoculum (the adsorption period was 300 minutes), while in Table III we wish to demonstrate the correlations between the size of inoculum and the length of incubation period, on the one hand, and the periods of time necessary for the appearance of the first CP changes and for the total destruction, on the other. It was sought how many days of incubation are necessary to achieve

25, 50, 75 and 100 per cent degeneration after inoculation of different doses of virus. The results are given in the left upper corners of the squares in Table III. It was also calculated how many days were necessary for a progress by

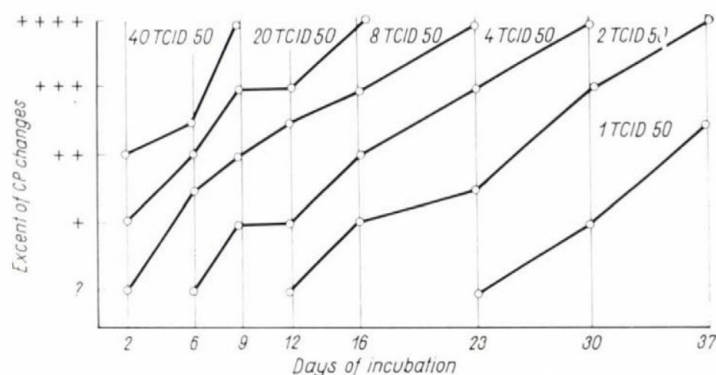


Fig. 7. The progress of CP changes in 7-days-old human amnion cell cultures infected with various doses of adenovirus 8/4

See footnote to Fig. 5.

Table III

Rate of degeneration in seven-day-old human amnion cell cultures infected with various doses of adenovirus 8/4

		Zone I		Zone II			
Dose of virus TCID ₅₀		Days necessary for					
		+		++		+++	
		degeneration					
Zone I	40	1	2 (1)	6 (4)	9 (3)	Zone II	
	20	2 (1)	6 (4) (4)	9 (3) (3)	16 (7) (7)		
Zone II	8	5 (3)	9 (3) (4)	16 (7) (7)	23 (7) (7)	Zone III	
	4	9 (4)	16 (7) (7)	23 (7) (7)	30 (7) (7)		
Zone III	2	16 (7)	25 (9) (9)	30 (7) (5)	37 (7) (7)		
	1	30 (14)	37 (12) (7)	NT	NT		

NT = not tested.

Explanation in the text.

one cross of the CP changes (one cross means the degeneration of 25 per cent of the cells). The results are seen in parentheses in the left lower corners of the squares. The figures in the right upper corners show how many more days were necessary to develop the same extent of degeneration when the actual dose was given instead of the nearest larger dose. In these experiments the adsorption period was 300 minutes.

On such an analysis the data shown in Table III may be divided into three zones. In zone I one day was required for a 25 per cent progress in the degeneration of cells and a 50 per cent reduction of the dose of virus prolonged the time necessary for the same extent of degeneration by only one day. In zone II both these indices rose to 3 or 4 days whereas in zone III 7 days were required for one-cross progress and a reduction of the dose by 50 or 60 per cent increased the requirement in time also by 7 days (or more).

Table III also shows that double the dose of virus generally resulted in one-cross more degeneration during the same period of time; a tenfold reduction of the dose, on the other hand, led to one-cross degeneration during the period being sufficient for the total dose to produce total destruction (++++).

Discussion

GINSBERG [12] demonstrated that the optimum period of adsorption for adenovirus type 1—4 was 4—6 hours. The present experiments have shown that adenovirus type 8 strains also require about 5 hours contact with the cells to produce the most rapid cell destruction. Although neither the estimation of CP changes nor their quantitative characterization by crosses are exact procedures, and the progress of the destruction is influenced also by other factors (*e.g.* the age of the culture) [13—15] it seems reasonable to state that the use of the optimal period of adsorption might improve the success of the isolation of virus also when type-8 virus is the pathogenic agent, even if the cultures are not washed after adsorption of the virus.

The present studies have shown that the inverse linear relation which, according to KJELLÉN [18] and others, exists in the case of certain adenovirus types between dose of virus and length of the latency period (the period up to the appearance of the first signs of degeneration) is not valid for type-8 with doses as small as were employed in the present studies. Within some limits, halving of the dose doubled the time required for one-cross degeneration; in a later phase, however, one-cross progress required more and more time. The slow progress of destruction after inoculation of small doses of virus might be a possible explanation of the difficulties in the isolation of the type-8 adenovirus from EKC cases; the conjunctival washings very probably contain only a few infective units [1, 5]. Consequently, prolongation of the period of observation and several blind passages may improve the isolation rate.

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A SEROLOGICAL STUDY OF THE INCIDENCE OF TICK-BORNE ENCEPHALITIS IN HUNGARY

By

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Summary. In the period 1958 to 1962, 1230 blood samples obtained from acute neurological cases have been tested for neutralizing antibodies against the tick-borne encephalitis virus. Out of 290 cases of encephalitis, 459 cases of meningitis, and 91 cases diagnosed as poliomyelitis or facial paresis, 25%, 13%, and 0%, respectively, proved to be positive. The incidence of antibodies increased parallel with the patients' age.

Incidence of antibodies was demonstrated in almost every region of Hungary.

Without virological examination a considerable part of the tick-borne encephalitis cases is registered as aseptic meningitis.

The neutralization test as carried out in HeLa cell cultures proved to be a reliable serological test.

The occurrence of tick-borne encephalitis in Hungary was proved in 1952, when two strains of the virus (KEm₁ and KEm₂) were isolated from *Ixodes ricinus* L. collected in the surroundings of a small country town which seemed to be an endemic focus of the illness. Both strains could be neutralized with convalescent sera of local patients suffering from meningo-encephalitis [1]. Subsequently FÖRNÖSI demonstrated by neutralization tests carried out in mice that tick-borne encephalitis occurred in further three counties.

Most recently, since compulsory country-wide vaccination of children against poliomyelitis by the live vaccine had been introduced, the incidence of paralytic illnesses has decreased. To throw some light on the aetiology of the still existing sporadic poliomyelitis like, as well as of other CNS diseases serum samples or, if possible, paired sera of such patients have been examined for neutralizing antibodies to tick-borne encephalitis. The tests were carried out in HeLa cell cultures according to the method described by LIBIKOVÁ and VILČEK [5].

Materials and methods

Blood samples. The 1230 serum samples, including 336 serum pairs, examined in this study were obtained from 840 neurological cases and sent to this Laboratory during the years 1958–1962 in order to be tested for enteroviruses or antibodies to tick-borne encephalitis. The sera of the patients who had proved positive for enterovirus were not tested for tick-borne encephalitis.

According to the preliminary clinical diagnosis the patients were classified into three groups, viz. (i) encephalitis, (ii) aseptic meningitis, and (iii) poliomyelitis and facial paresis. The sera were inactivated at 56° C for 30 minutes and stored at +4° C until tested.

Tissue cultures. HeLa cells grown in a medium containing 40 per cent human serum were trypsinized and suspended in a medium consisting of 10 per cent calf serum and 90 per cent Hanks' solution enriched with 0.5 per cent lactalbumin hydrolysate. One ml cell suspension containing 15,000 cells was placed in every test tube [5, 6].

Virus. The Hungarian strain of tick-borne encephalitis virus, which had been isolated and propagated in the mouse brain, was adapted to HeLa cells [6]. The virus already cytopathic for HeLa cells was re-inoculated into mouse brain and the material of the first mouse passage was suspended in rabbit serum to make a 20 per cent brain suspension. This suspension was distributed in vials and stored at -70°C . When tube cultures were prepared, the inoculum was added to the cell suspension at the time of the latter's distribution in tubes. In presence of virus the cells became attached to the glass wall and grew on it, but showed degeneration, namely shrinkage, after 2–7 days.

For the neutralization test 1:20-diluted sera were mixed with an equal volume of a virus suspension containing 100 CPD₅₀ of virus per 0.2 ml. The mixtures were kept at 37°C for 90 minutes and then inoculated into HeLa cultures, 0.2 ml per tube. Two cultures were inoculated with each of the mixtures. The cultures were observed for eight days after inoculation. A serum was regarded positive if at 1:20 dilution it prevented the cytopathic changes in both cultures. The positive sera were then tested in 1:100, 1:500 and 1:1000 final dilutions to estimate neutralization titres.

Results

Out of the 840 patients under study 134 had neutralizing antibodies to tick-borne encephalitis virus, but none of the 91 patients with the preliminary clinical diagnosis of poliomyelitis or facial paresis had such antibodies. In most of these cases the clinical course of the illness did not conform with poliomyelitis. In the seropositive cases the diagnosis was encephalitis or meningitis. Out of the 290 cases of "encephalitis" (group I of Table I) 25 per cent, while of the 459 "meningitis" cases (group II of Table I) only 13 per cent, proved to have antibodies. It is of interest that in the years 1959 and 1960 there was no difference between groups I and II in the percentage incidence of antibodies.

A comparison of the titres of acute-phase sera to those of convalescent specimens is shown in Table II. In 280 cases only acute-phase specimens,

Table I

Incidence of neutralizing antibodies against tick-borne encephalitis virus in sera collected from neurological cases observed in Hungary, 1958–1962

Year of illness	Encephalitis			Meningitis			Total		
	number of cases tested	positive		number of cases tested	positive		number of cases	positive	
		No	%		No	%		No	%
1958	34	6	17	7	1	14	41	7	17
1959	30	7	23	74	16	22	104	23	22
1960	64	14	22	84	20	23	148	34	23
1961	48	9	18	154	9	6	202	18	9
1962	114	36	31	140	16	12	254	52	20
1958–62	290	72	25	459	62	13	749	134	18

whereas in 197 cases only convalescent specimens were available. Out of these 16 and 54 sera, respectively, proved to contain antibodies. The number of serum pairs was 336; in 277 cases both specimens were negative. In 23 positive cases the antibody level was identical in the two specimens. A significant rise was shown by 29 serum pairs. In these 29 cases the diagnosis of tick-borne

Table II

Distribution of the sera by antibody titre to tick-borne encephalitis virus

		Titre* of the first blood sample						Total
		<20	20	50	100	≥1000	N. t.	
Titre of the 2nd blood sample	≥1000	4	2	3	5	13	29	56
	100	6	1	4	7	1	19	38
	50	8	1	3	1		2	15
	20						4	4
	<20	277					143	420
	N. t.	264	2	1	7	6		280
Total		559	6	11	20	20	197	813

* Reciprocals.

N. t. = not tested.

Table III

Distribution by age and sex of the patients tested

Sex	Age in year												Total		
	0—15			16—25			26—			Unknown					
	No. of cases	positive		No. of cases	positive		No. of cases	positive		No. of cases	positive		No. of cases	positive	
		No.	%		No.	%		No.	%		No.	%		No.	%
Males	143	14	10	87	21	24	124	41	33	33	4	12	387	80	21
Females	190	9	5	110	9	8	125	32	26	28	4	14	453	54	12
Total	333	23	7	197	30	15	249	73	29	61	8	13	840	134	16

encephalitis must be accepted; in the remaining 93 cases the antibodies might have originated from earlier infections.

Table III shows the age and sex distribution of the cases. The percentage incidence of antibodies grew parallel with age, it was higher for males than for females (21 and 12 per cent, respectively). The sex difference seemed to be more pronounced under 26 years of age than in the older group.

Fig. 1 shows the domiciles of the "encephalitis" and "meningitis" cases. Accordingly, the occurrence of tick-borne encephalitis has been revealed in

every county of Hungary, except four (I, II, V and XIV). However, from counties I and V only 2 and 3 cases, respectively, have been tested. Previously natural foci had only been known in four counties (III, X, XI and XIII).

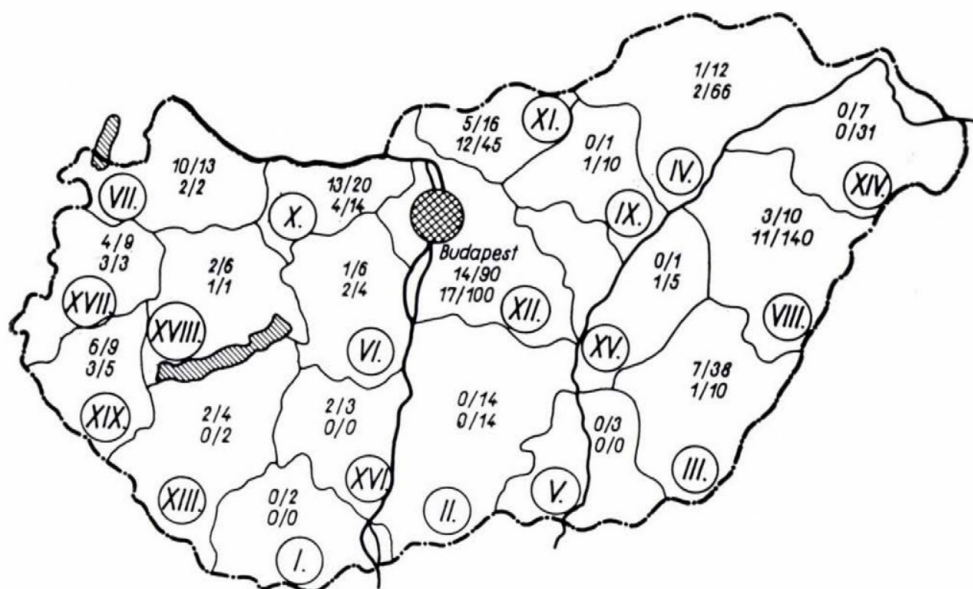


Fig. 1. Tick-borne encephalitis in Hungary, 1958—1962

Explanations: Upper fraction: encephalitis
Lower fraction: meningitis
Denominator: number of cases tested
Numerator: positive cases

Discussion

The present data have provided information on the geographical incidence of tick-borne encephalitis in Hungary, showing moreover that a considerable proportion of the cases diagnosed as encephalitis or aseptic meningitis is, in fact, tick-borne encephalitis. The respective percentages, 25 and 13 per cent, may be higher than the real percentage incidences, as the existence of antibodies without demonstration of an increase in titre may indicate an earlier infection. Moreover, the "encephalitis" cases included a few cases with the preliminary diagnosis of tick-borne encephalitis, and cases positive for enteroviruses were not tested. Thus, the cases were not sampled at random. On the other hand, the incidence of tick-borne encephalitis among the cases diagnosed as encephalitis or meningitis must be higher than 8.6 per cent (29 cases showed an increase in titre out of the 336 from which paired sera were available), since the cases which had antibodies in the "acute-phase" samples have been excluded from this calculation. Most of the "acute-phase"

samples were taken after the neurological symptoms had appeared, though this period may have represented the second wave of the illness. The first wave may escape attention or may be registered as a "summer cold", "meningism" or something else. In a case of laboratory infection [7] we found a high antibody titre as soon as the first neurological symptoms had appeared.

The present data have shown that tick-borne encephalitis is widespread in Hungary, especially in the wooded regions. It is unlikely that the three counties where antibodies have not been revealed are unaffected.

Our observation that men, especially those from 16 to 25 years of age, are affected to a higher extent than women of the same age, conforms with the literary data and is explainable by differences in occupation and mode of life.

The yearly fluctuation in the percentage of positive cases seems to be insignificant.

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THE EFFECT OF METHANOL ON THE GROWTH OF STREPTOMYCIN-DEPENDENT STRAINS OF *ESCHERICHIA COLI* IN STREPTOMYCIN-FREE MEDIA

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Summary. Inoculation of streptomycin-dependent strains of *Escherichia coli* into streptomycin-free media is well known to result in the occurrence of elongated forms. The appearance of these forms can be inhibited by methanol, other organic solvents, or sucrose. Under such conditions growth can be observed in the absence of streptomycin. The extent of growth depends on the media. The experimental results suggest that in streptomycin-dependent strains streptomycin plays a role in cell-wall synthesis and cell-division.

According to data in the literature [1] the streptomycin requirement of streptomycin-dependent bacterial strains is a specific characteristic, and streptomycin can only be substituted by certain structurally related compounds [2, 3].

The mutagenic effect of antibiotics has been determined by IYER and SZYBALSKI's agar diffusion test [4], the frequency of back-mutants in a streptomycin-dependent strain of *E. coli*. Methanol applied as a solvent was found to interfere with the detection of the mutagenic effect; in streptomycin-free media, growth was stimulated by this aspecific substance.

The observed phenomenon has been studied in detail and the results obtained are presented below. Some results have already been published [5].

Materials and methods

Strains. The following streptomycin-dependent strains were used in the experiments: *Escherichia coli* Sd-4-73 (cysteine-dependent), *Escherichia coli* J. A. 178 S. M., *Proteus mirabilis*, *Bacillus megaterium*.

Maintenance of strains. The streptomycin-dependent strains were maintained on slant agar containing 50 µg/ml streptomycin.

Media. Complete medium: beef heart extract, pH 7, with 0.5 per cent peptone and 0.5 per cent sodium chloride. As minimal medium, that of DAVIS and MINGIOLI [6] was used containing 0.5 µM of KH_2PO_4 . In case of *E. coli* strain Sd-4-74 the medium contained 25 µg/ml L-cysteine-hydrochloride. In some experiments the medium was supplemented with 0.5 per cent L-glutamic acid or 0.5 per cent acid-hydrolyzed casein.

Preparation of inocula. The inocula were prepared on a horizontal shaker at 37° C, in complete media containing in the case of *E. coli* and *Proteus mirabilis* 10 µg/ml, while in the case of *Bacillus megaterium* 50 µg/ml, streptomycin. After 18 hours of culturing the bacteria were washed twice with distilled water and further inoculations were carried out with cell-concentrations given in the experimental section.

The growth experiments were performed in test tubes in a 37° C incubator and the number of colony-formers was determined on agar plates containing 10 µg/ml streptomycin. The number of nondependent back mutants occurring in the cultures cultivated under different conditions was controlled by plating on streptomycin-free agar. In the evaluated experiments the frequency of back-mutants did not exceed the value of 10^{-4} .

Results

Effect of methanol on the streptomycin-dependent strain E. coli Sd-4-73 on complete medium. When a streptomycin-dependent *E. coli* strain is cultivated on streptomycin-free medium, multinuclear filamentous forms evolve after a few cell-divisions. If methanol was added to the cultures at different concentrations at the time of inoculation, with increasing the methanol concentration, in the 24-hour-old cultures the number of filamentous forms gradually decreased, falling to zero in the presence of about 2.0–2.5 per cent of methanol. A similar effect was observed with the use of some other solvents, e.g. ethanol,

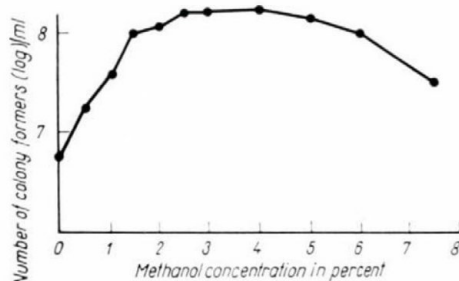


Fig. 1. Effect of methanol concentration on *E. coli* strain Sd-4-73. The experiments were carried out in complete medium. Inoculation: 2×10^6 cell/ml. The number of colony formers was determined in the 18th hour

n-propanol, iso-propanol and acetone. The change in colony former count is shown in Fig. 1. It is seen that the colony former count gradually increased until the methanol concentration had reached 2.5 per cent and it decreased above a methanol concentration of 5 per cent, owing to the toxic effect of the organic solvent.

Bacterial growth was studied in streptomycin-free media, and in the presence of 10 $\mu\text{g/ml}$ of streptomycin and 5 per cent methanol, respectively (Fig. 2). A stepwise decrease of the colony former count due to the decay of the filamentous forms could be observed in streptomycin-free media. Methanol produced a growth curve similar to that obtained in the presence of streptomycin. The effect of methanol did not depend on the quantity of the inoculum. If during growth the concentration of methanol was reduced by a tenfold or greater dilution of the medium, the increase of the colony former count was brought to a stop.

This effect of methanol was supported by the observation that the spreading the streptomycin-dependent strain on an agar plate containing 5 per cent methanol or 10 $\mu\text{g/ml}$ streptomycin resulted in the same number of colonies. The colonies grown on methanol-containing plates are streptomycin-dependent. (The 5 per cent methanol had been added when pouring the

agar; contained 4.5 per cent methanol on immediate determination, while after 24 hours 1.5 per cent, after 48 hours 0.9 per cent methanol was present.)

If streptomycin is added to the cultures grown on streptomycin-free media for 18 hours, normal bacilli are produced after 2–3 hours from the

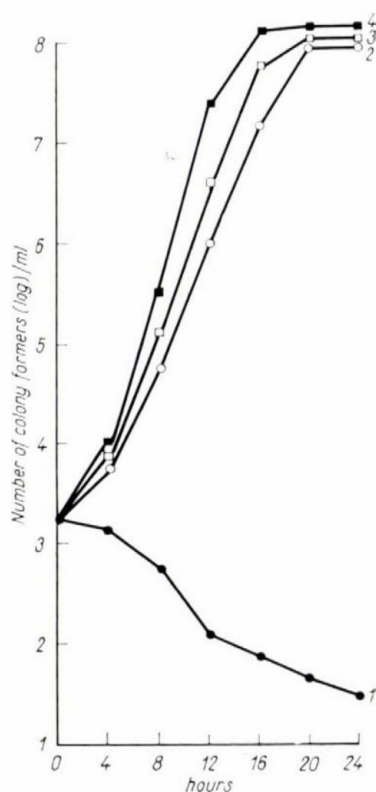


Fig. 2. Effect of methanol concentration on *E. coli* strain Sd-4-73 in complete medium

- 1: streptomycin-free medium
- 2: 5 per cent methanol-containing medium
- 4: 10 μ g/ml streptomycin-containing medium
- 3: 5 per cent methanol and 10 μ g/ml streptomycin-containing medium

initially present elongated forms. With long filamentous forms, however, this transformation was generally not quite complete.

Growth of streptomycin-dependent E. coli strain Sd-4-73 on minimal medium. The effect of methanol on the growth of the streptomycin-dependent *E. coli* strain has been studied on minimal medium. It was observed that, though filamentous forms evolve in streptomycin-free cultures, slight if any loss in colony former count occurs. On the administration of streptomycin, a curve similar to that obtained on complete nutrient was observed, while on the addition of methanol the colony former count increased in 24 hours

only by one order of magnitude, although the production of filamentous forms had completely stopped. On adding glutamic acid to the minimal nutrient, an increase of the colony former count by two orders of magnitude occurred in the same time interval in the presence of methanol. Methanol had a strong inhibitory effect on growth in minimal medium containing streptomycin (Fig. 3).

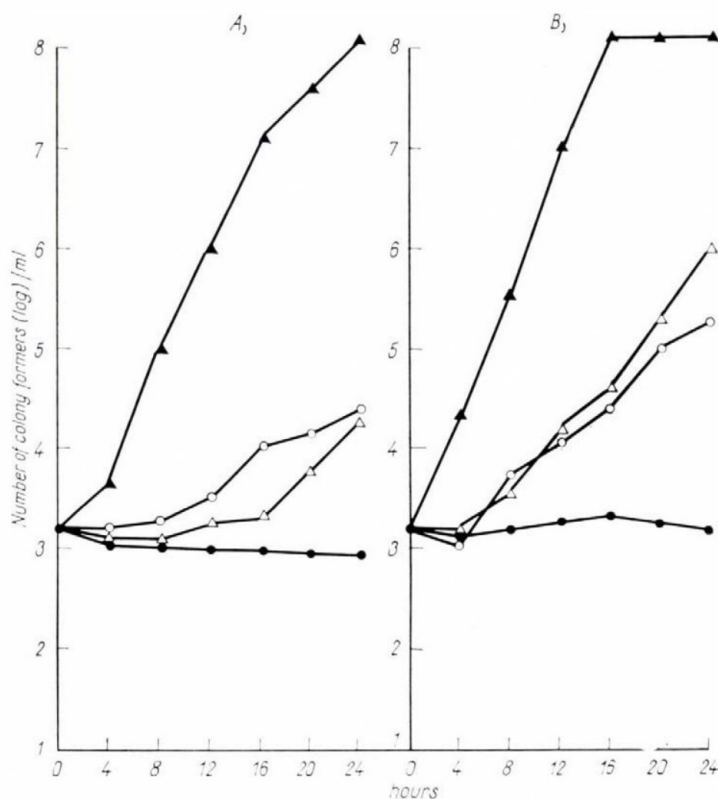


Fig. 3. Effect of methanol on *E. coli* strain Sd-4-73 in minimal (A) and 0.5 per cent glutamic acid containing (B) medium

- streptomycin-free medium
- 5 per cent methanol-containing medium
- ▲—▲ 10 µg/ml streptomycin-containing medium
- △—△ 5 per cent methanol and 10 µg/ml streptomycin-containing medium

If acid-hydrolyzed casein was added to the minimal nutrient and the concentration of the inoculum was gradually decreased, the occurrence of filaments, then of plasmoptysic forms and finally sphaeroplasts and their ghosts could be observed in 18-hour cultures, accompanied by a diminution by several orders of magnitude of the colony former count. At certain inoculum-concentrations, cultures predominantly consisting of sphaeroplasts were obtained.

The quantitative data of these experiments varied markedly, depending on the circumstances applied. In the presence of streptomycin, abnormal forms failed to appear on this medium, while methanol reduced their production.

Effect of methanol on other bacteria in streptomycin-free complete medium. Essentially similar results were obtained with *E. coli* strain J. A. 178 S. M.

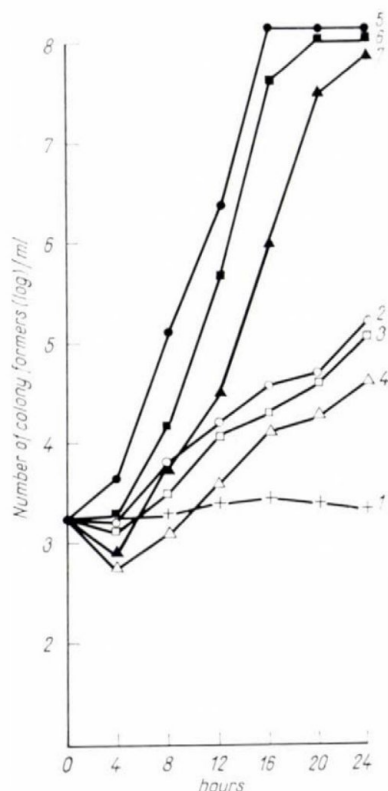


Fig. 4. Effect of sucrose on *E. coli* strain Sd-4-73 in minimal medium containing 0.5 per cent L-glutamic acid

- 1: basal medium
- 2: 6 per cent sucrose-containing medium
- 3: 12 per cent sucrose-containing medium
- 4: 20 per cent sucrose-containing medium
- 5: 10 μ g/ml streptomycin-containing medium 6 per cent sucrose + 10 μ g/ml streptomycin-containing medium
- 6: 12 per cent sucrose + 10 μ g/ml streptomycin-containing medium
- 7: 20 per cent sucrose + 10 μ g/ml streptomycin-containing medium

On the addition of methanol the filamentous forms evolved in streptomycin-free media at first decreased then practically disappeared. This required an 5 per cent methanol concentration, but the methanol tolerance of this strain is also higher. At the optimum methanol concentration the same colony former

count could be attained as in the presence of streptomycin, though for this a longer time interval was required than in the case of *E. coli* strain Sd—4—73.

With *Bacillus megaterium* and *Proteus mirabilis* no production of filaments was observable in streptomycin-free media and cell-division was not stimulated by methanol.

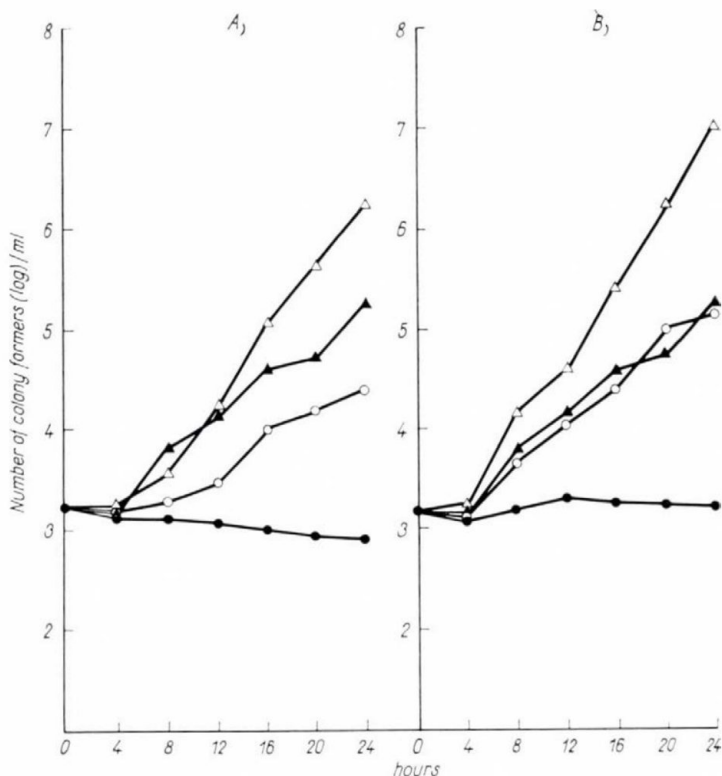


Fig. 5. Combined effect of sucrose and methanol on *E. coli* strain Sd—4—73 in minimal (A) and 0.5 per cent glutamic acid containing (B) medium

- basal medium
- ▲—▲ 6 per cent sucrose-containing medium
- 5 per cent methanol-containing medium
- △—△ 6 per cent sucrose- and 5 per cent methanol-containing medium

Effect of sucrose on E. coli strain Sd—4—73 in streptomycin-free media.

It has been mentioned that by using an appropriate inoculum concentration, a great number of sphaeroplasts could be observed in media containing casein hydrolyzate. An attempt was made to stabilize the sphaeroplasts in sucrose. The experiments showed that a sucrose concentration of more than 3 per cent not only stabilized the sphaeroplasts but also prevented their production, *i.e.* exhibited an effect similar to that of methanol. The effect of sucrose was then studied in minimal medium completed with glutamic acid (Fig. 4).

By means of 6 per cent sucrose the number of cell-divisions can be enhanced, and in the microscopic picture the number and length of the elongated forms was reduced, though they did not entirely disappear. Applied at the above concentration, sucrose had no effect on the growth and on the morphologic form in media containing streptomycin. Increasing the concentration of sucrose to over 12 per cent, elongation was completely prevented and growth was inhibited, even in media containing streptomycin.

Similar results were obtained on complete medium; the production of filaments, however, did not cease even in 20 per cent sucrose. The effects of methanol and sucrose were compared in experiment shown in Fig. 5. It is to be seen that the two effects are summed up.

Discussion

It has been observed that on the addition of methanol growth is initiated in two *E. coli* strains of different origin, and the elongated, filamentous forms characteristic of the absence of streptomycin disappear. By means of methanol the constriction of the evolved filamentous cells of strain Sd-4-73 can be produced; the sphaeroplast formation observable under certain circumstances is also reduced. During growth in the presence of methanol the streptomycin-dependence is maintained. The effect may also be achieved with several alcohols, acetone, and also with sucrose at high concentration. No such phenomenon could be observed with streptomycin-dependent strains of *Bacillus megaterium* and *Proteus mirabilis*, where the lack of streptomycin did not result in the occurrence of elongated forms.

The observed phenomenon cannot be interpreted in terms of the theory put forward by SPOTTS and STANIER [7] according to which streptomycin promotes protein synthesis through ribosome activation. Although ERDŐS and ULLMANN [8], and SPEYER *et al.* [9] have shown that in the case of streptomycin-resistant strains streptomycin does not inhibit the incorporation of amino acids into the protein *in vitro*, the verification of the streptomycin-dependence of this process was not successful [10]. Whereas SPOTTS [11] does not consider the disturbance of the cell-wall synthesis as proven in the different streptomycin-dependent strains, the occurrence of abnormal forms in the absence of streptomycin seems to favour this possibility.

ANAND and DAVIS [12] called the attention to the disturbance of cell-membrane synthesis in streptomycin-sensitive *E. coli*. This effect of streptomycin resulting in the increase of permeability, can be inhibited by chloramphenicol.

LANDMAN and BURCHARD [13] attributed an important role to streptomycin in cell-wall synthesis and cell-division and suggested that a semi-

autonomous system present in the cell-membrane is responsible for both functions.

Our experiments seem to support the latter conception and in our opinion they unequivocally prove that in the case of streptomycin-dependent strains of *E. coli* the absence of streptomycin results in a marked disturbance of cell-wall synthesis, and the occurrence of elongated forms must primarily be attributed to the failure of the cell-membrane to constrict. Both cases can be influenced by physical (osmotic, surface active) effects so that no, or only a partial, inhibition of growth occurs.

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HETEROTYPIC HAEMAGGLUTINATION INHIBITION IN THE ADENOVIRUS GROUP

By

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Summary. Paired sera of 40 patients with epidemic keratoconjunctivitis, and further 208 human blood specimens were tested for haemagglutination-inhibiting antibodies to adenovirus types 8, 9 and 10. A close correlation was found between the type-8 antibody titres and the titres to type 9, and a less marked correlation between these and the anti-10 titres. Haemagglutination-inhibition tests carried out with type sera prepared in rabbits have supported the existence of the heterotypic reactions.

Parallel with the discovery of hitherto unknown adenovirus serotypes more and more heterotypic serological reactions, most of them based on the neutralization test, have been reported [1–5]. ROSEN [6] when describing the haemagglutination-inhibition (HI) test for adenoviruses listed the types giving cross reactions, there are, however, few data on heterotypic HI antibodies in human sera [7, 8]. In the course of the keratoconjunctivitis epidemic that occurred in Budapest 1961–1962, we observed by the HI test hitherto undescribed cross reaction between adenovirus types 8 and 9, on the one hand, and type 10, on the other [9]. This phenomenon was investigated in the present study in human blood specimens and in type sera prepared in rabbits.

Materials and methods

Viruses. The adenovirus type strains 1–7 and 9–16 were kindly supplied by Dr. J. VILČEK, the "Ijima" strain of the type 8 virus, from Dr. E. JAWETZ. Another type 8 strain isolated by us from a case of epidemic keratoconjunctivitis (EKC) [10] was used for testing paired sera. The strains were cultivated and purified as described earlier [11].

Immune sera. Rabbits were immunized with the above strains as published elsewhere [11]. The immune sera were treated with argil, and stored at -20°C until used. If necessary, the sera were absorbed with the erythrocytes to be used in the HI test.

To confirm the results obtained with our own type-8 serum, parallel tests were performed with a rabbit immune serum kindly supplied by Dr. HUEBNER's laboratory. The techniques applied in the treatment of human blood specimens, in the preparation of erythrocyte suspensions, and in the HI test have been described in detail [12, 9].

Results

Human paired sera. Forty serum pairs obtained from patients with EKC were tested.

Table I shows the acute-phase titres and the titre increase in the sera of the 40 patients. Each of these became seropositive to types 8 and 9, while

Table I

Percentage distribution of the HI titres to three adenovirus types and of the degree of the increase in these titres in the paired sera of 40 patients with EKC

Adenovirus type	Serum	HI titre*			Increase		
		<4	4-8	≥16	no	twofold	fourfold and over
8	acute	75.0	7.5	17.5	—	5***	95
	convalescent**	—	7.5	92.5			
9	acute	72.5	12.5	15.0	—	17.5	82.5
	convalescent	—	12.5	87.5			
10	acute	85.0	5.0	10.0	37.5	12.5	50.0
	convalescent	37.5	12.5	50.0			

* Reciprocals.

** Taken 4-6 weeks after onset of EKC.

*** To another type 8 strain these, too, showed a fourfold rise.

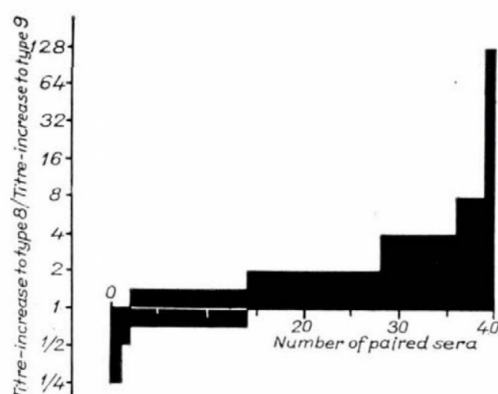


Fig. 1. Relation of type 8 HI-titre increases to type 9 increases in paired sera of patients with EKC

15 per cent of the acute-phase sera and 62.5 per cent of the convalescent specimens were positive against type 10. An at least fourfold rise in titre was found in 95, 82.5 and 50 per cent of the paired sera when tested against types 8, 9 and 10, respectively.

The correlation between these antibody reactions was also examined.

Fig. 1 shows that in two serum pairs the antibody response to type 9 was in one case twice, in another fourtimes greater than that to type 8; in 12 cases the titre increase to the two viruses was of the same degree, whereas in the remaining 26 cases the anti-8 titre showed the greater rise. Out of the 40 serum pairs 25 displayed an antibody response to type 10, too (Fig. 2).

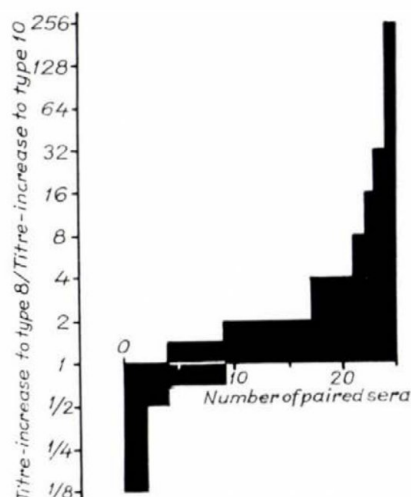


Fig. 2. Relation of type 8 HI-titre increases to type 10 increases in paired sera of patients with EKC

In 5 cases the type 8 and type 10 titres rose to the same degree, in 4 cases the anti-10, in 16 cases the anti-8 titre showed the greater increase.

Human single blood specimens. Two hundred and eight sera were tested each of which contained HI antibodies to at least one of types 8, 9 and 10. The results are illustrated in Fig. 3.

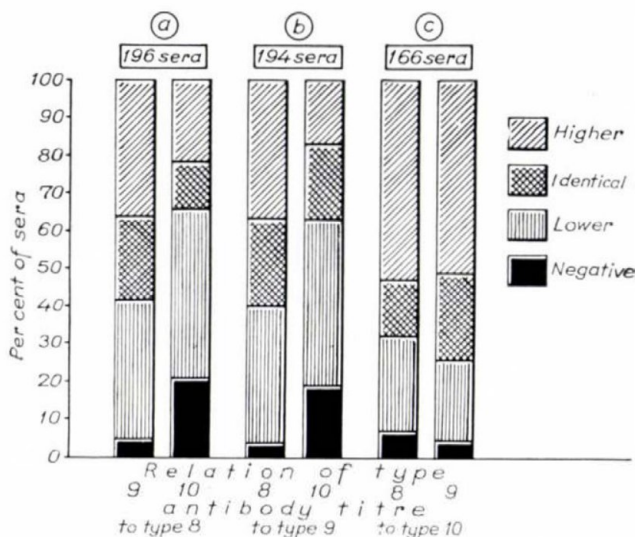


Fig. 3. (a) Percentage distribution of adenovirus type 9 and 10 titre values in relation to the adenovirus 8 titres of 196 sera each positive against type 8
 (b) Percentage distribution of adenovirus type 8 and 10 titre values in relation to the adenovirus 9 titres of 194 sera each positive against type 9
 (c) Percentage distribution of adenovirus type 8 and 9 titre values in relation to the adenovirus 10 titres of 166 sera each positive against type 10

Accordingly, 196 sera inhibited adenovirus type 8; of these 186 (95 per cent) inhibited type 9, too; 44 gave equal titres, while 70 and 72 samples gave higher and lower titres, respectively, to type 9 than to type 8. Out of the 196 sera positive to type 8, 155 (81 per cent) inhibited type 10, too, but more than a half of these gave a lower titre to type 10.

Of the 208 sera 194 inhibited adenovirus type 9; 96 and 81 per cent of these also inhibited types 8 and 10, respectively. The titre distribution was very similar to that detailed above.

A hundred and sixty-six sera gave the HI reaction to adenovirus type 10. A great part of these (93 and 95 per cent) also inhibited types 8 and 9, respec-

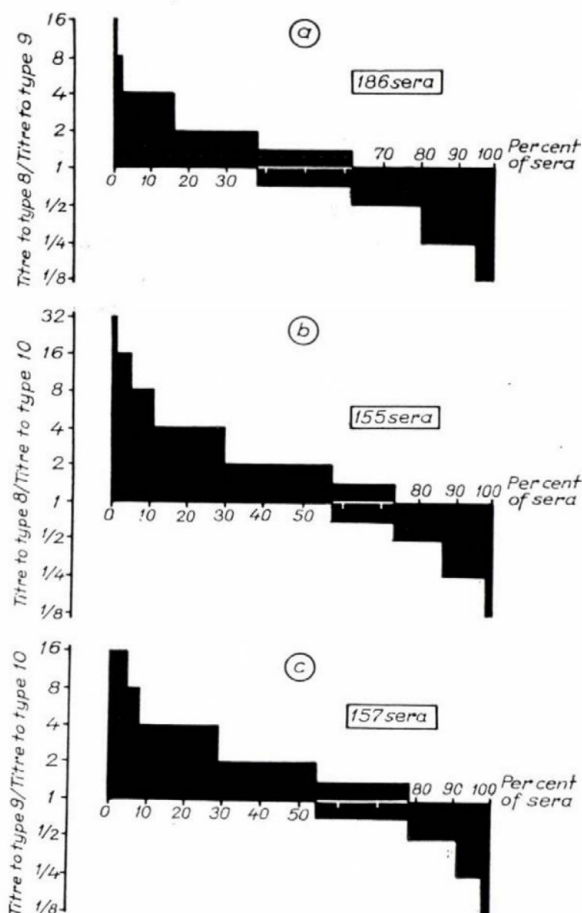


Fig. 4. (a) Percentage distribution of 186 sera positive both to adenovirus type 8 and 9 according to the ratio of titres to type 8 per type 9
 (b) Percentage distribution of 155 sera positive both to adenovirus type 8 and 10 according to the ratio of titres to type 8 per type 10
 (c) Percentage distribution of 157 sera positive both to adenovirus type 9 and 10 according to the ratio of titres to type 9 per type 10

tively. Only about a quarter of these sera gave lower titres to types 8 and 9 than to type 10.

A detailed comparison is shown in Fig. 4. It is seen that the titre distribution for types 8 and 9 was about the same while the anti-10 titres were, in general, somewhat lower.

Table II

HI titres to adenovirus type strains of adenovirus type sera prepared in rabbits

Type serum	HI titre* to adenovirus type									
	3	6	7	8	9	10	11	13	15	16
1	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—
3	256	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—
6	—	2048	—	—	—	—	—	—	—	—
7	—	—	512	—	—	—	128	—	—	—
8	—	—	—	4096	1024	32	—	—	—	—
9	—	—	—	1024	4096	128	—	—	—	—
10	—	—	—	—	64	16384	—	—	—	—
11	—	—	1024	—	—	—	16384	—	—	—
13	—	—	—	—	—	—	—	16384	—	—
14	—	—	128	—	—	—	1024	—	—	—
15	—	—	—	—	—	—	—	—	1024	—
16	—	—	—	—	—	—	—	—	—	512

* Reciprocals.

— = Negative at 1:8 dilution.

Rabbit sera: Table II shows the cross titration results of the type sera prepared against adenovirus types 1 to 16. It should be noted that our strains type 1, 2, 4, 5 and 14 gave too low HA titres to carry out the HI test against these viruses. Cross reactions were observed among types 7, 11 and 14 and among types 8, 9 and 10. However, type 8 virus was not inhibited by the anti-10 serum.

Discussion

Several investigators have reported on cross reactions among different types of adenoviruses using immune sera prepared in animals or human sera. Thus, cross neutralization was found between types 4 and 7; 11 and 7; 7 and 7a; 7, 11 and 14; 15, 4 and 9; 7, 3 and 14; 4, 3 and 7; 16 and 4; 25, 10 and 15;

26, 9 and 27, etc. [4, 5, 13–16]. Cross neutralization between types 8 and 9 has been demonstrated with rabbit immune sera [1, 17], and with human sera in connection with EKC [18.]

Virus types 9 and 10 showed a weak cross reaction with each other in the experiment of GRAYSTON *et al.* [1].

Using the HI test, ROSEN [6] found cross reactions between types 7 and 7a; 7, 11 and 14; 8 and 9; and 10 and 19. Others reported on cross reaction between types 22 and 23. Peculiarly between these two strains no cross neutralization could be demonstrated. Certain cross reactions, on the other hand, are demonstrable only by the neutralization test [20].

Data on cross HI reaction between adenovirus types 8 and 9, on the one hand, and type 10, on the other were found only in the report of ROSEN *et al.* [21]. The anti-9 rabbit immune serum of these authors inhibited type 10 virus, but not *vice versa*. The cross HI test between types 8 and 9 is well-known. In human sera, however, heterotypic reactions among the three types studied by us have to our knowledge never been demonstrated.

The data summarized in Table II agree well with ROSEN's observations as regards the cross reactions among types 7, 11 and 14; he, too, found that the type 8 virus was inhibited by type 9 heterotypic serum, but not by the type 10 one. On the other hand, each of the type 8, 9 and 10 antisera inhibited both the 9 and 10 types of adenoviruses. It need not be emphasized that for every serum the homologous titre was the highest.

We have described in detail the immune response of our EKC patients [9]. Here only the cross reactions will be discussed. The antibody level to both of the types 8 and 9 rose in all the 40 paired sera tested; the increase was significant, *i.e.* at least fourfold, in 95 and 82.5 per cent of the cases, respectively. To type 10, 20 patients showed a significant response, in further 5 cases the increase was only twofold (Table I).

Taking into account (i) the cross reactions in the type sera, (ii) our failure to isolate type 9 or 10 strains from conjunctival washings during the epidemic period, and (iii) the lack of antibody response to types 11 and 16 [9], the rise of the anti-9 and anti-10 antibody titres in the EKC sera should be considered as heterotypic reactions.

The quantitative analysis of the immune responses (Figs. 1 and 2) agreed with the literary data. DAWSON *et al.* [18] found higher titres to type 9 than to type 8 (the latter having been the pathogenic agent) in the sera of three of the seven members of a family suffering from EKC; in one case the titre was the same against both types.

The titres of the 208 human blood specimens alone would not prove the existence of cross reactions, but considering that (i) more than half of the donors (107) were affected by the EKC epidemic; (ii) that the other specimens, too, were taken during the epidemic; (iii) that no correlation was found with

other adenovirus types, the demonstration of HI titres against types 9 and 10 in most of the 208 sera, support the conclusion drawn from the examination of the paired sera.

The data in Figs. 3 and 4 suggest that, though there exists a cross HI reaction between types 8 and 10 this is not so marked in its consistency, or quantitative respects than that between types 8 and 9. This might explain why the phenomenon had not been discovered before.

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EFFECT OF IODOCASEIN FEEDING ON THE ANTITOXIN TITRE OF ANIMALS USED IN SERUM PRODUCTION

By

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Summary. (i) Feeding of iodocasein caused a threefold increase in the diphtheria antitoxin titre of a horse which had previously been subjected to a 21 month course of continuous immunization.

(ii) During a 13 month period of immunization the average staphylococcal antitoxin titre of 4 sheep fed with iodocasein was significantly higher (maximum 256 per cent) than that of the 6 control animals.

(iii) As to tetanus antitoxin titre, among 22 cattle no significant differences were revealed between the treated and control animals.

(iv) Of 20 horses immunized with tetanus toxin for 6 months, the antitoxin titre in 10 animals fed with iodocasein was continuously higher than in the control group; significant differences were noted for a short period only. The maximum difference between the average titre of the two groups was 45 per cent.

(v) The rise in antitoxin titre occurred generally after 6—8 weeks feeding of iodocasein.

(vi) The antibody production-promoting effect of iodocasein varied with the species of animal. The increase was definite in sheep, moderate in horses and variable in cattle.

(vii) Iodocasein treatment exerted a uniform promoting effect on antitoxin production, regardless whether the animals were immunized with staphylococcus, diphtheria or tetanus toxin.

Antibody production, as a special kind of protein synthesis, is associated with intermediary metabolism. As indicated by various observations, the two systems are governed by many common principles and both are under the co-ordinating control of neuro-endocrine centres. Vitamins, especially those of group B, are able to increase the synthesis of antibodies [1—6]. A protein deficient diet or the lack of certain essential amino acids may decrease the production of immune-globulins [3—11]. If the necessary amino acids are not provided during immunization, the antibodies are built up at the expense of other protein fractions. In a number of hyperimmunized animals the albumin and globulin fractions decrease parallel with the increase of the specific antibody-containing T-globulin fraction [12—13]. In an impaired condition, immunization is often unsuccessful. The response to the same antigen is highly influenced by the species, age, condition, constitution and hormonal balance of the immunized individual. Thus, the amount of antibodies formed and the rate of production are only partly influenced by the immunization process. Successful immunization is closely associated also with the metabolic condition of the individual.

GÜNTHER has attempted to explain the double dependence of antibody production by the "Fließband" theory [14—17]. According to his conception,

immune-globulins may be regarded as altered normal globulins produced in the body in a manner analogous to the industrial straight line production of complicated work pieces. At the beginning of the process the constituents of proteins, the amino acids are placed onto the moving band. The different sites of work are represented by various enzymes and at a certain stage by the antigen. During operation the whole protein or specific antibody molecule is built up from amino acids. The amount of the produced material depends on the running speed of the moving band. The antibody titre increases when higher amounts of antigen are given, or the time elapsing between injections is shortened. According to GÜNTHER's theory in these cases the enhanced antibody production is associated with the increase of the speed of the "moving band", that is, of globulin metabolism. In addition, every aspecific factor capable of increasing or decreasing the rate of protein metabolism, will cause a respective increase or decrease in the amount of produced antibodies.

Although the outlined hypothesis awaits additional experimental confirmation, there are some data to support its correctness.

(i) In passive immunization the excretion half-life of antibodies in adult individuals of the same species is practically constant. When metabolism is increased by the administration of thyroxine, the half-life of the injected gamma globulin decreases [18].

(ii) The effectiveness of active immunization is greatly enhanced by thyroxine. As compared to the control group, LONG [19] showed within 4 months a 2.4-fold increase in antibody titre when guinea pigs received diphtheria toxoid simultaneously with thyroxine. MILCU [20] working with rats immunized by typhoid and paratyphoid antigens found that antibody titres in the thyroxine-treated group exceeded by 229 per cent the titres measured in the control group. Feeding of iodinated protein to animals immunized with the same antigen for 24 days caused a 368 per cent rise.

(iii) Italian authors [21] observed an increase of thyroid function and thyrotropin excretion in 29 patients suffering from acute infectious diseases.

(iv) In cold blooded animals the blood level of antibodies decreases with the decrease of body temperature and basal metabolism [22, 23].

(v) Thyroxine contributes to the synthesis of coenzyme A [25, 26]. In the absence of this enzyme antibody production considerably decreases. Thyroxine increases the number of lymphocytes which play a part in antibody production.

Thus, many observations confirm the important role of thyroxine and metabolism in antibody production. This finding may have a practical value in the evaluation of diagnostic serological tests or in the preparation of immune sera, etc. The practical use of these results is hindered by the fact that the principles of the effect of metabolism-promoting substances are unknown.

The parallelism between antibody production and basal metabolism, the optimal dose of thyroxine, its effect on antibody formation by various species, the association between the quality of antigen and antibody production in thyroxine-treated animals and the question whether thyroxine should be given in the inductive or progressive phase of antibody production, still await elucidation. In the present study answers were sought to these problems. The examinations were carried out on animals used for large-scale serum production. To increase metabolism, iodinated casein was given, because in suitable preparations this substance contains about 0.5 per cent thyroxine and is much less expensive than crystalline thyroxine.

Materials and methods

Experimental animals. Merino female sheep 2–2½ years of age, Hungarian red coloured oxen 3–5 years of age, pure breed mares and castrated horses 4–10 years of age were used.

Iodocasein was manufactured by "G. Richter" Pharmaceutical Co., Budapest. The total iodine content of the preparation was 9.4; the thyroxine content, approximately 0.5; the fat content, 0.68 per cent.

Antigens. Tetanus toxin and toxoid were prepared as described by SURJÁN [27]. The potency of the preparation was between 20–30 units/ml.

Diphtheria toxin and toxoid of 40–50 Lf/ml potency were prepared in Linggood medium.

Staphylococcal toxin and toxoid were prepared by filtration of liquid digest medium cultures of staphylococci. The titre of the preparation was 500–2000 DHM, BU 0.8–1.4/ml [28].

Immunization. The animals were given at monthly intervals two basal doses of toxoid adsorbed to aluminium hydroxide gel. Five days after the second dose 20 ml liquid toxoid was given. Then at 5 day intervals gradually increased amounts of the liquid toxoid were injected. After 9 days rest, graded doses of the corresponding toxin were administered at 4 day intervals. Blood samples were taken from the jugular vein 5 days after the last booster dose at 21–22 day intervals. From each sheep 0.5 l blood was withdrawn. From cattle and horses 5 and 6 l samples were taken on each of two consecutive days, respectively.

Feeding with iodocasein. Sheep readily consumed the substance dispersed on the forage. Each sheep in the fed group received a daily dose of 3.5 g for 10 weeks. The animals lost considerable weight and developed tachycardia and functional heart murmurs. Iodocasein feeding was therefore suspended for 4 weeks until these symptoms disappeared. Treatment was then continued with 1.5 g daily doses and hyperimmunization was begun simultaneously.

For cattle, daily doses of 30 g iodocasein were intended. Because the forage mixed with the drug was refused by these animals, the weekly dose was given in two parts in an aqueous suspension and at the same time hyperimmunization was performed.

For horses, the calculated dose was 20 g. As these animals also refused the iodocasein-containing forage, they were fed half the weekly doses treaded with water into balls. These were readily swallowed when placed onto the radix of the tongue. Simultaneously, hyperimmunization was performed.

Measurement of antitoxin. Tetanus antitoxin was titrated in white mice weighing 18 g each, by the biological method at the L+/10 level. The Copenhagen standard served for comparison. Diphtheria antitoxin titre was measured by the Ramon method based on initial flocculation.

Staphylococcal antitoxin was titrated by measuring the inhibition of the haemolysis of rabbit erythrocytes at the LH/100 level, as described by SZATHMÁRY [29].

Evaluation of results. Titres obtained in various immunized groups were analyzed by use of STUDENT'S *t* test.

Results

Horses immunized with diphtheria toxin. Two horses that had been immunized for 21 months with diphtheria toxin were used for the experiments. The animal with the lower titre was given 5 g iodocasein daily between November 10 and December 26. Between December 26 and January 8 the daily dose was increased to 15 g, between January 9 and February 1, to 30 g. The horses were bled on February 4. The antitoxin titres are presented in Fig. 1. It is seen that iodocasein feeding caused a considerable rise in the antitoxin titre.

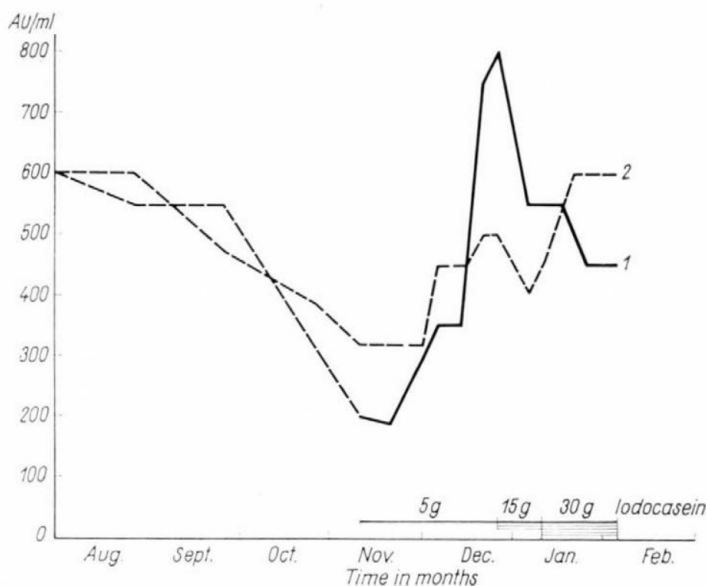


Fig. 1. Diphtheria antitoxin titre of horses 1. Fed animal; 2. Control animal

After some weeks, despite the continued iodocasein feeding, the antitoxin level fell below that measured in the control animal. As both horses were extremely overstrained by the immunization courses lasting nearly 2 years, these experiments were considered to provide only orientating data.

Sheep immunized with staphylo toxin. Prior to immunization the serum of 3 out of 10 sheep contained staphylo-antitoxin. These 3 animals were left as controls, as for them the first injection of staphylo toxin was in reality a secondary stimulus. Of the remaining 7 sheep 3 were included in the control group, while 4 animals were fed from the beginning with iodocasein. After 7 months, from April 30 onward, each control animal received daily 1.5 g of iodocasein. The average antitoxin titres measured during the experiment are shown in Fig. 2. The antitoxin level in animals fed for 8 weeks with iodocasein was superior to that in the control animals, which at first exhibited

higher titres. It is seen that when iodocasein doses were decreased, the stimulating effect also decreased, but the antitoxin level never fell below that found in the control group. When, because of alterations in the quality of antigen or due to non-specific factors, a decreased immune response was obtained the fall in titre was parallel in both groups. As doses of 3.5 g daily caused weakness, feeding was discontinued between November 15 and December 10. Subsequently daily doses of only 1.5 g were given. Between November 15 and December 5 no immunizing injections were administered. Fig. 2 shows that, due to this break, the average titre considerably decreased in both groups.

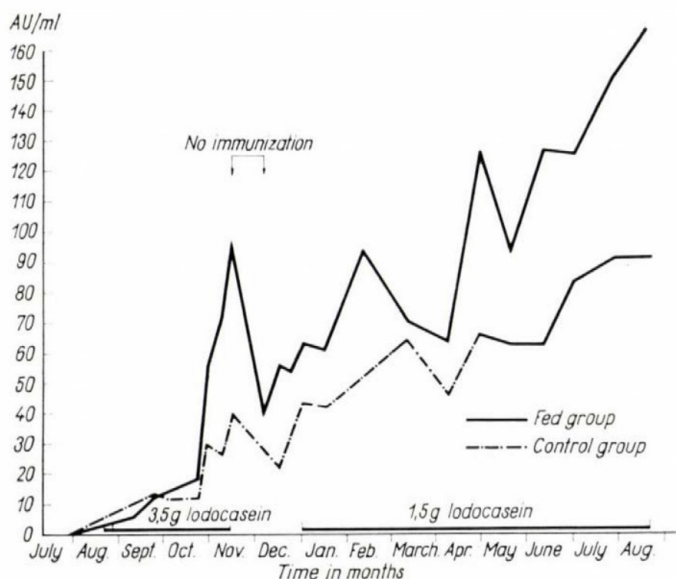


Fig. 2. Average staphylococcal antitoxin titre of sheep

In the treated group, the members of which received no iodocasein in this period, the fall in titre was more than 100 per cent. The mean data evaluated by STUDENT's method gave the following results.

In regard to all titres obtained during the experimental period (July 27—April 29), the difference between the 4 treated and 6 control animals was $t_{29} = 2.09 = < 5\%$. This result indicates that the iodocasein treated animals exhibited significantly higher titres. In those control animals which received iodocasein from April 30 onward, the difference between the average titre of 5 blood samples taken before iodocasein feeding and the average titre of blood samples taken during iodocasein-feeding, was $t_8 = 2.3 = 5\%$. Accordingly, after the beginning of iodocasein treatment the titres rose significantly. In respect of the corresponding samples taken between January 13 and April 29

and May 19 and August 18 from animals fed from the beginning with iodocasein, t_8 was $1.83 = >10\%$ (not significant). Thus it is evident that iodocasein feeding started as late as 7 months of immunization was still able to give rise to significantly raised antitoxin levels.

Cattle immunized with tetanus toxin. Of 22 cattle, 14 were fed with iodocasein from the beginning of immunization; 8 animals were left as controls.

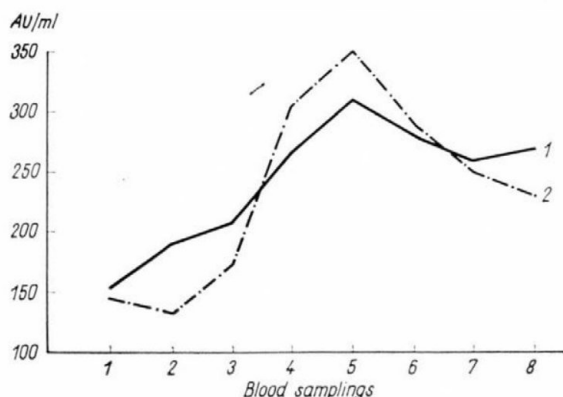


Fig. 3. Average tetanus antitoxin titre of cattle. 1. Fed group; 2. Control group

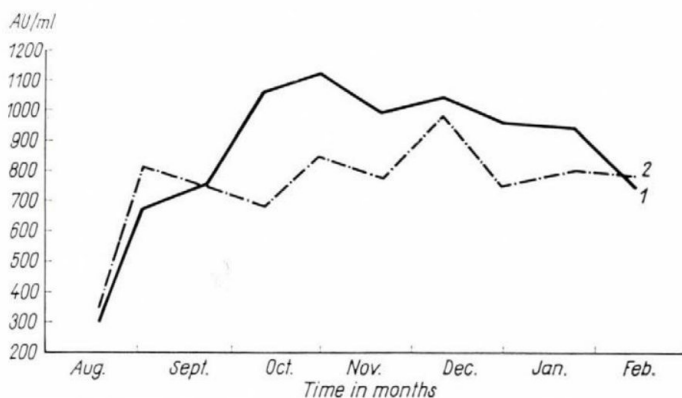


Fig. 4. Average tetanus antitoxin titre of horses 1. Fed group; 2. Control group

The average of titres is shown in Fig. 3. Both groups exhibited poor immune responses. The antitoxin level found in the first three blood samples was higher in the treated group. The 4th, 5th and 6th samplings gave opposite results. Considering the variable results and that the animals had lost weight, iodocasein feeding was discontinued after the 6th blood sampling. The inconstant data were unsuitable for statistical analysis.

Horses immunized with tetanus toxin. After a preliminary immunization the antitoxin titres in 20 horses were determined. The animals were then

divided into two groups, so that the initial average titre in the control group was 20 per cent higher than in the group to be fed with iodocasein. This substance was given simultaneously with the beginning of hyperimmunization. Immunization lasted for one half year. During that time 10 blood samples were taken at intervals of 3 weeks. The results are presented in Fig. 4. Although the average titres in the fed group were always higher, the difference was significant only with the 4th and 6th blood samplings (Table I). The immune respons-

Table I

Difference in the tetanus antitoxin titre of iodocasein-treated and control horses

Blood sample No.	t	%	Difference
4th	2.10	< 5	+
5th	1.57	> 10	—
6th	2.10	< 5	+

Difference: + = significant, — = not significant.

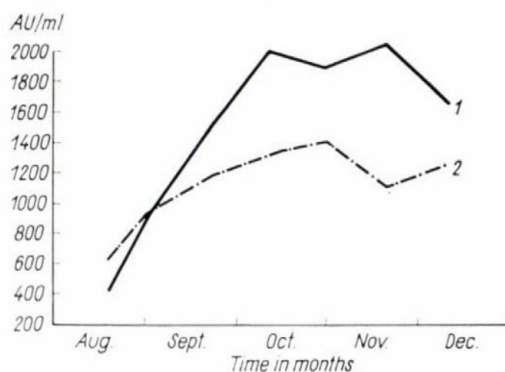


Fig. 5. Average tetanus antitoxin titre of 8 most reactive horses. 1. Fed group; 2. Control group

es showed high individual variations in both groups. In our opinion the more susceptible the animal to the antigenic stimulus, the more reliable data are yielded by the immunological experiment. Therefore, the average titres of 4 more definitely reacting horses of each group were plotted separately (Fig. 5). When STUDENT's method was applied, the difference between the mean titres measured on 7 occasions of sampling was $t_{31} = 2.28 = < 5\%$ (significant for the whole period). Similarly to the sheep, both groups of horses reacted to a weaker antigenic stimulus with a decreased antibody production. The difference between the fed and control groups, however, was likewise left

unaltered (e.g. 6th blood sampling, November 20). After one half year of immunization the titre decreased in both groups, iodocasein had lost its stimulating effect and the fed animals' titre gradually fell to the level of the control group. After the 10th sampling the animals with lower titres were bled in both groups. No alteration was revealed in the antitoxin titre of the remaining further immunized horses. Summarizing the results for horses, it has been evident that, as compared to the controls, the group fed with iodocasein showed a higher immune response in the first half year of immunization. Significant differences were revealed only in well-reacting horses.

Discussion

In our experiments iodocasein feeding caused definite, transient or durable, stimulation of antibody production in animals belonging to various species.

The rise in antibody titre ran parallel with the consumed amount of iodocasein. Taking the average titre in the control animals as 100 per cent, the maximum titre in sheep fed with 3.5 g daily doses of iodocasein, was 256 per cent. When the daily dose was decreased to 1.5 g, the rise in the treated group amounted to 193 per cent.

When iodocasein feeding and immunization were discontinued simultaneously, the average titre fell considerably. The decrease was more rapid in the fed animals, that is, in those having an increased basal metabolism. By extrapolating the data presented in Fig. 2, it may be calculated that the half-life of the average antitoxin titre is about 48–52 days in the control group, while only 20–24 days in the treated group. DIXON *et al.* [18] obtained similar results with passive immunization.

Iodocasein feeding of sheep and of horses and cattle treated with tetanus toxin was begun simultaneously with hyperimmunization. Horses immunized with diphtheria toxin and 5 sheep left previously as controls for staphylotoxin immunization received iodocasein only after a longer period of immunization. The titre in both kinds of animal rose definitely. The effect persisted in horses only for 2 months. In sheep the rise was less than that obtained in animals fed with iodocasein from the beginning. A definite increase of antibody production occurred in all three animal species only 6–8 weeks after starting iodocasein treatment. A similar observation was made when iodocasein was administered to animals previously subjected to long-lasting hyperimmunization.

The optimal dose of iodocasein varied with the animal species. In sheep, 1.5–2 g, in horses and cattle, 20–25 g daily promoted antibody production without impairing the animals' condition. Higher doses (3.5 g for sheep and 30 g for cattle) caused after some weeks a rapid loss of weight and tachycardia.

The effectiveness of iodocasein feeding highly depends on the species of animal. As compared to the control groups, LONG [19] obtained in guinea pigs a 240 per cent rise in titre. The rise observed by MILCU [20] in rats was 229 per cent. In the present studies the best effect was reached in sheep. In cattle the titres showed a considerable difference in favour of the fed group only on one occasion, at the 2nd blood sampling. It would appear that the antibody-producing apparatus reacts better to metabolism-increasing agents smaller than bigger animals.

This consideration may be associated with the higher metabolism rate of small animals. The half-life of passively administered gamma globulin is 1.9 days in the mouse, 5 days in the guinea pig and rabbit, 8 days in the dog, 13 days in the adult man and 21 days in cattle [18]. Our consideration is further supported by the finding that iodocasein was most effective in animals reacting more actively to immunization, or, in terms of GÜNTHER's theory, in animals in which the speed of the "moving band" of globulin metabolism is higher.

Iodocasein treatment seems to be effective in immunization by a wide range of antigens. Uniformly good results were obtained by LONG with diphtheria toxoid by MILCU with typhoid and paratyphoid vaccine, and in the present experiments with staphylococcal, diphtheria and tetanus toxoids. In the course of immunization with tetanus toxoid of horses and cattle only the former responded with an increased antibody production when fed with iodocasein.

The alteration of the given amount of antigen caused generally a parallel increase or decrease in antibody titres.

Our results have confirmed the reports on the beneficial effect of metabolism-increasing drugs in antiserum production. In addition, the results more or less support GÜNTHER's "moving band" theory. In large scale serum production the method appears advantageous for increasing the antibody titres.

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THE INFLUENCE OF TEMPERATURE ON THE MULTIPLICATION OF THE PR8 STRAIN OF INFLUENZA A VIRUS AND ON THE INTERFERON PRODUCTION BY THE VIRUS INFECTED CELLS

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Summary. The multiplication of the PR8 strain and the simultaneous interferon production has been studied at various temperatures in the embryonated egg.

(1) During the first 12 hours after infection the rate of multiplication was somewhat higher at 39° C and 40° C than at 35° C. At higher temperatures, however, the multiplication was inhibited even during this early phase.

(2) The highest titres were demonstrated at 24 hours, irrespective of temperature. The virus yield was optimal at 35° C and it was inversely related to the temperature between 39° C and 42° C.

(3) At 35° C interferon was first demonstrable at 24 hours and its titre was rising from this time up to the 96th hour. The initial production was increased between 39° C and 41° C, but at 40° C and 41° C the production stopped after 24 hours.

(4) At 39° C, 40° C and 41° C the 24-hour interferon titre was twice, fourfold, and eightfold, respectively, that obtained at 35° C.

(5) In eggs incubated at 41° C for 24 hours and at 35° C thereafter the interferon production did not stop at 24 hours.

The results are discussed in relations of the altered cell metabolism at supraoptimal temperatures and of the significance of the observed phenomena in the recovery from viral infections.

Since the discovery of interferon by ISAACS, LINDENMANN and VALENTINE [1, 2], the circumstantial factors of the production of this agent has been studied in several respects. It has been demonstrated that the non-specific factors that influence viral multiplication influence interferon production, too. Thus, low temperature or reduced oxygen tension inhibit [1, 3], whereas mild acid milieu stimulates the production of interferon [4].

Nonspecific factors, e.g. temperature, may play an important role in the defence mechanism of the organism. Since viral infections are often accompanied by fever, the question arose [19] how supraoptimal temperatures would influence the ratio of interferon production and virus multiplication. In the present experiments the PR8 strain of influenza virus type A was studied in this respect.

Materials and methods

Viruses. The PR8 strain of influenza A virus and the Sendai strain of parainfluenza A virus were maintained in the allantoic cavity of 11-day-old chick embryos. The harvested allantoic fluids were stored in sealed ampoules at -20° C until used.

Cultivation of virus. An adequate number of embryonated eggs pre-incubated for 12 days was inoculated into the allantoic cavity with 100 egg-ID₅₀ of the PR8 strain each and

re-incubated at 35° C, 39° C, 40° C, 41° C or 42° C. At intervals of 12, 24, 48, 72, and 96 hours, six eggs were removed from each of the incubators and placed in the refrigerator (+4° C) for 12 hours. The allantoic fluids were then harvested and of the samples obtained from the individual eggs of the same group equal volumes were mixed. Each pool was then divided into two parts and kept at -20° C until tested for interferon and infectious virus.

Titration of virus. The infectious virus was titrated by HORVÁTH's [5] roller drum technique; the haemagglutinin titre was determined with TAKÁTSY's *Microtitrator* [6]. ID_{50} was calculated by the REED—MUENCH formula [7] and expressed in $\log_{10}$ units.

Titration of interferon. Allantoic fluids were dialyzed against glucosol [5] and heated at 65° C for 30 minutes; then a twofold dilution series was prepared from each of the samples in HORVÁTH's medium [5] and the dilutions were distributed in vials (0.5 ml per vial) in each of which one drop of surviving chorioallantoic membrane fragments had been placed. Four such cultures were inoculated with each of the dilutions and rotated in the roller drum until the indicator virus (100–1000 CAM- ID_{50} of the Sendai virus to every culture) had been 24 hours later. The vials were then rotated again for 60 hours. Finally, each of the culture media were tested for haemagglutination, using TAKÁTSY's technique [6]. The quantity of interferon is expressed in "interferon-titre" units, i.e. in the highest dilution being able to prevent the production of demonstrable haemagglutinin in at least two of the four vials.

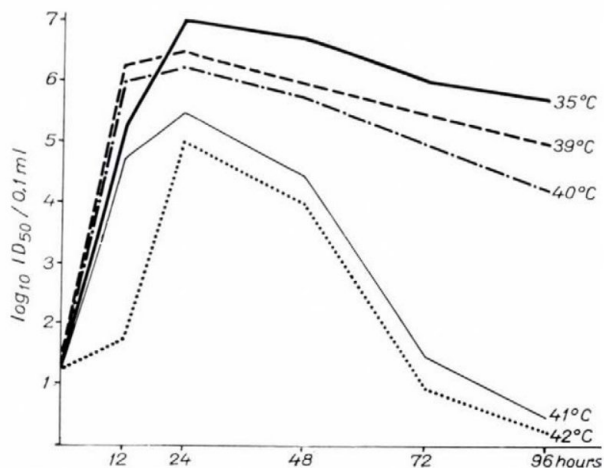


Fig. 1. Growth of PR8 strain in embryonated egg at different temperatures

Experimental

The experimental procedure was essentially the same as those used by WAGNER [8] and by LEVINE [9]. Infectious virus and interferon in the allantoic fluids of eggs incubated at the given temperature were titrated for various periods after inoculation with the PR8 strain.

Fig. 1 shows marked differences in virus growth at different temperatures. During the first 12 hours the rate of multiplication appears to be slightly higher at 39° C and 40° C than at 35° C, whereas at 41° C and 42° C virus growth is inhibited even during this early phase of infection. The highest titres are shown at the 24th hour, irrespective of the temperature of incubation. At this time the virus titre was inversely related to the temperature within the range 35° C to 42° C. At 42° C the maximum was about one 100th of that

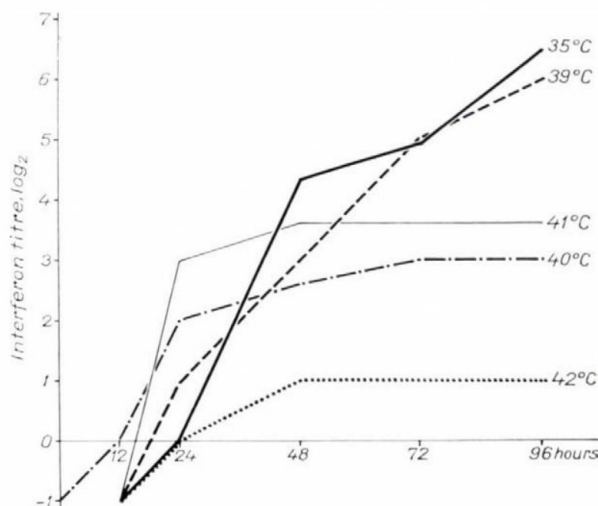


Fig. 2. Interferon production in PR8-infected embryonated eggs at different temperatures

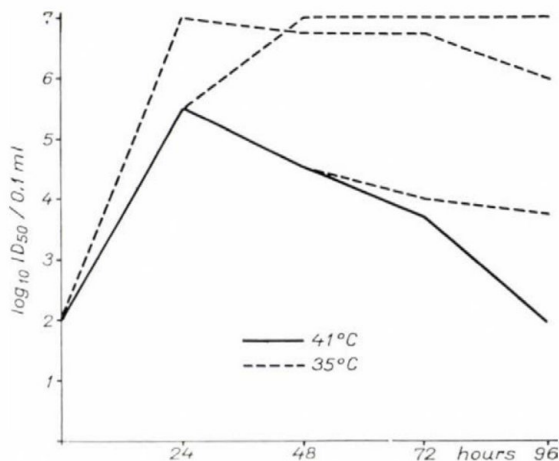


Fig. 3. Growth of PR8 strain in embryonated eggs incubated at 35°C after pre-incubation at 41°C

obtained at 35°C. From the 24th hour on the titres tended downward, at higher temperatures faster than at 35°C, so that at 96 hours hardly any virus was demonstrable in the allantoic fluids of the eggs incubated at 41°C or 42°C.

Fig. 2 shows the interferon curves. The interferon concentration is expressed in log₂ units. At 35°C interferon appeared at 24 hours and its level increased up to the 96th hour, when the experiment was finished. During the first 24 hours more interferon was produced at 39°C, 40°C, and 41°C than at 35°C. The 24-hour titre was the highest in the allantoic fluids of the eggs

incubated at 41° C (1 : 8); simultaneous titres at 35° C, 39° C, 40° C, and 42° C were 1 : 1, 1 : 2, 1 : 4, and 1 : 1, respectively. Thus, during the first 24 hours an equal amount of interferon was produced at 42° C and 35° C.

In the experiments illustrated in Figs. 3 and 4 eggs were incubated at 41° C for 24 or 48 hours after infection, whereafter incubation was continued at 35° C. It is seen that if the temperature had changed after 24 hours, the initially poor virus production did not stop, so that by 48 hours the infective

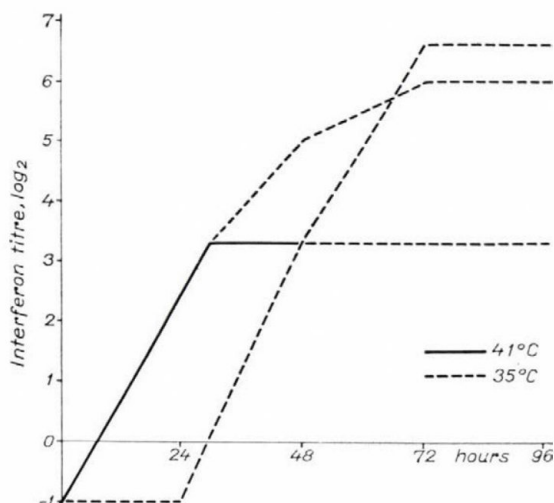


Fig. 4. Interferon production in PR8-infected embryonated eggs incubated at 35° C after pre-incubation at 41° C

titre attained that shown by the allantoic fluids of the eggs kept at 35° C during the whole period of incubation. In the same eggs an intensive interferon production was shown during the first 24 hours and production did not cease up to the 72nd hour, when the titre approximated that shown by the eggs incubated at 35° C during the whole period. In contrast, in the eggs consistently incubated at 41° C interferon was not produced after the 24th hour.

The results clearly show that interferon production greatly depends on temperature, but is hardly dependent on the actual titre of the infectious virus.

Discussion

Viral multiplication is greatly influenced by nonspecific factors, *viz.* temperature, pH, oxygen tension, etc. According to ENDERS [10], nonspecific factors play an important role in the defense mechanism of the macro-organism during the initial period of infection, *i.e.* before specific antibodies appear. In recovery from viral infections ISAACS [11] attributes an outstanding role to interferon.

Several authors have examined the effect of temperature on viral reproduction. LWOFF and LWOFF [12] demonstrated strain differences in the thermosensitivity of the development of polioviruses. Supraoptimal temperatures inhibit the initial phase of viral reproduction. WALKER and BORING [13], LWOFF *et al.* [14, 15] and GROMAN *et al.* [16] found that animals kept at 36° C survived Coxsackie or poliovirus infections at a higher rate than those kept at room temperature. BANG and LUTRELL [17] as well as LWOFF [18] have pointed out in their reviews that supraoptimal temperatures inhibit viral reproduction both *in vivo* and *in vitro*, and raise the survival rate of infected animals. However, there are only few data available on the relationship between temperature and interferon production. Recently, RUIZ-GOMEZ and ISAACS have demonstrated that chick embryo fibroblast cultures infected with some cytopathogenic viruses (Chikungunya, Kumba, O'nyong-nyong or Newcastle Disease virus) produce increased amounts of interferon at supra-optimal temperatures.

Like the authors cited we also wished to study interferon production as the function of viral reproduction. For this reason we induced interferon production with live virus. Raising the temperature from 35° C to 39, 40 or 41° C was found partially to inhibit the growth of the PR8 strain in the allantoic cavity of the chick embryo. Interferon production, on the other hand, was stimulated by temperatures from 39° C to 41° C during the first 24 hours after infection. During the later phases interferon production was the same at 39° C as at 35° C, while at 40° C and 41° C no appreciable amounts of interferon were produced. Nevertheless, interferon production continued if after 24 hours at 41° C the incubation was continued at 35° C.

The causes of these phenomena are not clear. Alteration of cell metabolism at higher temperatures may be an important factor. A possible increase in the production of incomplete virus might exert an indirect effect. Later, on the second day after infection, the impairment of cell metabolism, due probably to early damages in the synthesis of histons and basic proteins, may inhibit and even stop interferon production.

At 42° C there was no initial stimulation of interferon production observable, and the interferon titre was at a low level during the whole period of experiment. By this temperature the embryos were obviously injured from the 48th hour on.

Similar relations may exist in the human organism, too. The fact that influenza cases with high fever are often followed by a fast recovery supports the existence of an interaction between interferon production and fever.

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BREAKDOWN OF AMINO-ACIDS BY ENTEROBACTERIACEAE: ITS USE AS A ROUTINE DIAGNOSTIC TEST

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Summary. A synthetic medium has been prepared for the routine examination of deaminase activity of enteric bacteria. Experiments with some strains indicated that the test might be useful for differentiation purposes.

In a previous paper it has been reported that acid peptone water may be alkalized by enteric bacteria [3]. Further examinations indicated that this alkalization was due to ammonia liberated in the course of amino acid deamination [2]. In the present studies it has been shown that this reaction can be used for differentiation purposes.

Materials and methods

Cultivation. The culture medium recommended by KAMEDA *et al.* [1] was modified as follows. In an Erlenmeyer flask 1 g NH_4Cl , 1 g K_2HPO_4 , 0.5 g MgSO_4 , 1 g amino acid, 0.01 g nicotinamide, 0.1 ml 1 per cent CaCl_2 , 0.05 ml 1 per cent ferric ammonium citrate and 12 ml BCP indicator were dissolved in 1 litre distilled water.

Preferably L-amino acids were used. When only DL substances were available, these were applied in 0.2 per cent concentration. Prior to addition to the medium cystine was dissolved in *N* NaOH (1 g in 10 ml). BCP indicator was prepared by dissolving 2 g brilliant cresol purple in 50 ml *N*/5 NaOH and making up the volume to 1 litre. Some amino acids (cystine, tyrosine and tryptophan) dissolved only when steamed in ARNOLD's apparatus for one half hour.

The solutions were adjusted to pH 4.6 with *N*/10 HCl or *N*/10 NaOH at room temperature. After filtration through filter paper, sterilization was carried out at 115° C for 15 minutes. The solutions were left to cool at room temperature, then their pH was subsequently checked and corrected when it was below 4.7 or over 4.9. After pH correction the solutions were resterilized for one half hour in ARNOLD's apparatus.

The medium was dispensed in 200 ml portions in Erlenmeyer flasks and stored at room temperature until used (1 to 4 weeks). On the day of the examination the medium was distributed in 5 ml amounts into clear, sterile test tubes (16 mm by 160 mm). Inoculation was performed from one day agar cultures with an amount of bacteria somewhat larger than that usually picked up by a needle-point. Incubation lasted for 6 days at 37° C. Readings were performed daily. The results were classified as (i) negative, "—", when the medium remained acid throughout the incubation period, that is, no change in colour was observed; (ii) positive, "+", when bluish, blue or violet coloration indicating alkalization appeared within 4 days; and (iii) late positive, "(+)", when alkalization occurred after 4 days. Sometimes after 6 days the medium was of an intermediate colour (greyish). In such cases incubation was prolonged for two additional days. Strains giving alkaline reaction during this period were included in the late positive group. Otherwise the strains were classified as negative.

The cultures used in the present work were as described in reference [3]. Maintenance of the cultures was likewise unaltered.

Table I
Decomposition of amino acids by enteric bacteria

Group (subgroup, type)	No. of strains	Glycine pH 4.9			Alanine pH 4.6			Serine pH 4.7			Cysteine pH 4.6			Cystine pH 4.7			Threonine pH 4.6			Valine pH 4.7		
		+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>E. coli</i>	55	—	1	54	45	1	9	47	4	4	—	—	55	26	14	15	6	3	46	—	—	55
<i>Klebsiella</i>	15	5	8	2	15	—	—	15	—	—	15	—	—	14	1	—	—	—	15	—	—	15
<i>Citrobacter</i>	10	—	—	10	10	—	—	10	—	—	3	3	4	4	4	2	3	—	7	—	—	10
<i>Cloaca</i>	10	5	3	2	7	—	3	10	—	—	10	—	—	6	2	2	2	—	8	—	—	10
<i>Serratia</i>	6	6	—	—	6	—	—	6	—	—	5	1	—	—	—	6	3	—	3	—	—	6
<i>S. paratyphi A</i>	1	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1
<i>S. paratyphi B</i>	25	11	3	11	25	—	—	18	5	2	2	10	13	16	7	2	9	5	11	—	—	25
<i>S. typhi murium</i>	70	33	37	—	68	2	—	67	3	—	60	10	—	64	6	—	10	34	28	—	—	70
<i>S. kunzendorf</i>	1	—	—	1	—	—	1	1	—	—	—	—	1	—	—	1	—	1	—	—	—	1
<i>S. typhi</i>	25	5	—	20	—	—	25	.	.	.	—	—	25	—	—	25	—	—	25	—	—	25
Other salmonellae ¹ ...	8	3	—	5	8	—	—	8	—	—	5	3	—	7	1	—	1	2	5	—	—	8
Arizona	10	4	2	4	9	1	—	2	—	8	5	—	5	8	2	—	—	—	10	—	—	10
<i>Sh. flexneri I—3</i>	34	—	—	34	2	3	29	—	—	34	—	—	34	—	—	34	—	—	34	—	—	34
<i>Proteus</i> ²	37	—	—	37	—	—	37	—	—	37	—	—	37	—	—	37	—	—	37	—	—	37
<i>Providencia</i>	10	—	—	10	1	1	8	2	—	8	1	—	9	—	—	10	—	—	10	—	—	10

¹ *S. derby*, *S. stanleyville*, *S. bovis morbificans*, *S. enteritidis* and *S. anatum*.

² *P. vulgaris*, *P. mirabilis* and *P. morganii* 10 strains each; *P. rettgeri*, 7 strains.

Results

First, elaboration of a relatively simple method suitable for the large-scale investigation of deamination, was attempted. Among the various media used in preliminary experiments, amino acid solutions supplemented with sodium chloride and indicator, and adjusted to an acid reaction, gave satisfactory results. This medium, however, required larger inocula and repeated preliminary washing of the culture in saline. Finally, the method described under "Materials and methods" was accepted for routine testing, because, apart from the necessity of a careful adjustment of the pH, it was technically simple and the results were to read easily.

A further purpose of the experiments was to determine whether the method was suitable for the biochemical differentiation among various Enterobacteriaceae groups. The following amino acids were tested for decomposition: glycine, alanine, serine, cysteine, cystine, threonine, valine, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid, glutamic acid, glutamine, arginine, lysine and ornithine. The number of strains investigated was 317 (Tables I, II, III).

Table II
Decomposition of amino acids by enteric bacteria

Group (subgroup, type)	No. of strains	Phenylalanine pH 4.8			Tyrosine pH 4.8			Tryptophan pH 4.8			Histidine pH 4.6		
		+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>E. coli</i>	55	—	—	55	—	—	55	22	15	18	—	—	55
<i>Klebsiella</i>	15	—	—	15	4	1	10	—	—	15	15	—	—
<i>Citrobacter</i>	10	—	—	10	1	1	8	—	—	10	8	8	1
<i>Cloaca</i>	10	—	—	10	—	1	9	—	—	10	7	—	3
<i>Serratia</i>	6	—	—	6	6	—	—	3	—	3	6	—	—
<i>S. paratyphi A</i>	1	—	—	1	—	—	1	—	—	1	—	—	1
<i>S. paratyphi B</i>	25	—	—	25	—	—	25	—	—	25	—	—	25
<i>S. typhi murium</i>	70	—	—	70	—	—	70	—	—	70	—	—	70
<i>S. kunzendorf</i>	1	—	—	1	—	—	1	—	—	1	—	—	1
<i>S. typhi</i>	25	—	—	25	—	—	25	—	—	25	—	—	25
Other salmonellae ¹ ..	8	—	—	8	—	—	8	—	—	8	—	—	8
Arizona	10	—	—	10	—	—	10	—	—	10	—	—	10
<i>Sh. flexneri</i> 1—3	34	—	—	34	—	—	34	—	—	34	—	—	34
<i>Proteus</i> ²	37	—	—	37	—	—	37	—	—	37	—	—	37
<i>Providencia</i>	10	—	—	10	—	—	10	—	—	10	6	2	2

¹ *S. derby*, *S. stanleyville*, *S. bovis morbilligans*, *S. enteritidis* and *S. anatum*.

² *P. vulgaris*, *P. mirabilis* and *P.morganii*, 10 strains each; *P. rettgeri*, 7 strains.

The data are not analyzed in detail, as in our opinion the results are not sufficient for drawing final conclusions. Certain enteric bacteria seem to give consistently positive reactions in some amino acids but completely fail to attack others. *E.g.* *E. coli* decomposed aspartic acid but not lysine and cysteine, *Sh. flexneri* was inactive, and any of the examined strains split valine.

Table III
Decomposition of amino acids by enteric bacteria

Group (subgroup, type)	No. of strains	Aspartic acid pH 4.7			Glutamic acid pH 4.6			Glutamine pH 4.9			Arginine pH 4.6			Lysine pH 4.8			Ornithine pH 4.6		
		+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-	+	(+)	-
<i>E. coli</i>	55	55	—	—	42	8	5	52	—	3	—	—	55	—	—	55	—	—	55
<i>Klebsiella</i>	15	15	—	—	15	—	—	15	—	—	12	—	3	—	—	15	5	6	4
<i>Citrobacter</i>	10	10	—	—	10	—	—	10	—	—	6	—	4	—	—	10	—	—	10
<i>Cloaca</i>	10	10	—	—	10	—	—	8	—	2	9	—	1	—	—	10	6	1	3
<i>Serratia</i>	6	6	—	—	6	—	—	6	—	—	6	—	—	3	—	3	6	—	—
<i>S. paratyphi A</i>	1	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1
<i>S. paratyphi B</i>	25	18	4	3	18	7	—	18	3	4	18	—	7	5	—	20	25	—	—
<i>S. typhi murium</i>	70	68	2	—	45	10	15	68	2	—	—	—	70	—	—	70	70	—	—
<i>S. kunzendorf</i> .	1	1	—	—	—	—	1	—	—	1	—	—	1	—	—	1	—	—	1
<i>S. typhi</i>	25	2	1	22	—	—	25	—	—	25	—	—	25	—	—	25	—	—	25
Other salmonel- lae ¹	8	8	—	—	8	—	—	8	—	—	4	2	2	4	—	4	6	—	2
<i>Arizona</i>	10	9	1	—	8	2	—	9	1	—	4	—	5	3	—	7	4	—	6
<i>Sh. flexneri</i> 1—3	34	—	—	34	—	—	34	—	—	34	—	—	34	—	—	34	—	—	34
<i>Proteus</i> ²	37	9	—	28	12	1	24	—	—	37	—	—	37	—	—	37	—	—	37
<i>Providencia</i> . . .	10	10	—	—	10	—	—	—	—	10	—	—	10	—	—	10	—	—	10

¹ *S. derby*, *S. stanleyville*, *S. bovis morbificans*, *S. enteritidis* and *S. anatum*.

² *P. vulgaris*, *P. mirabilis* and *P. morganii*, 10 strains each; *P. rettgeri*, 7 strains.

Discussion

As shown in our previous experiments [2], alkalization in amino acid-containing media is due to a deamination of the substrate. Alkalinity is caused by the liberated ammonia. When, as in the present experiments, small inocula are applied, alkalization may thus be regarded as an indicator of multiplication. This has been confirmed in earlier investigations [3]. The shift of the pH is always preceded by the appearance of turbidity in the medium. Multiplication may take place without alkaline reaction when the assimilation of the amino acid occurs without the formation of basic products or if ammonia is produced

in amounts unsufficient to attain the relatively high pH range needed to cause a change in the indicator's colour.

The present results do not correspond to various known, widely applied biochemical reactions, such as the decarboxylase tests described by MØLLER and CARLQUIST, the phenylalanine deaminase test of HENRIKSEN, and the tryptophan deaminase test of SINGER and VOLCANI. The discrepancy may be explained by the influence of even slight changes on the enzyme activity of micro-organisms. The pH of the medium is especially important. In Table II it is seen that at pH 4.8 tryptophan is not decomposed by *Proteus* or *Providencia* strains. However, as it will be presented in a subsequent paper, these bacteria give positive alkali reactions in tryptophan medium adjusted to higher pH values.

It should be noted that no attempts were made to evaluate the differential diagnostic value of the method. Therefore, from the present experiments only the following conclusions can be drawn. (i) The simple method of deaminase activity determination of enteric bacteria may be included in routine diagnostic work. (ii) Certain enteric bacteria consistently decompose some amino acids, but not others. (iii) There are differences in the deaminase activity of various enteric bacteria. In view of the present data, it seems advisable to carry out wide-range investigations as to the applicability of the method for differential diagnostic purposes.

It is noteworthy that the media may be rendered alkaline by micro-organisms other than enteric bacteria. Reactions was observed in media inoculated with staphylococcal strains or contaminated by fungi.

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AUXOTROPHS OF *BACILLUS ANTHRACIS*

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Summary. The acapsulogenic mutant of *Bacillus anthracis* strain Vollum yielded 186 mutants, which, as compared to the parent strain, exhibited increased nutritional requirements. While at 37° C the parent strain grew suboptimally on a medium containing amino acid mixture (acid hydrolysate of casein supplemented with tryptophan and cystine) and thiamine, the mutants exerted the same degree of growth in the presence of additional metabolites only. Among the isolated 186 mutants, 99 purine and 57 pyrimidine dependent strains were encountered. Different vitamins were needed by 25 mutants (biotin, PABA, nicotinamide and riboflavin). Growth factors required by 5 further mutants were not identified.

More than half of the thymine and biotin dependent strains needed these substances only at 37° C; at 26° C their nutritional requirements were similar to those of the parent strain.

GLADSTONE has described that *Bacillus anthracis* needs in addition to salts and glucose various amino acids and glutamine as minimal requirement for its growth [1] and that an adequate proportion of the necessary amino acids was essential for the multiplication of this organism. Vitamin-free casein hydrolysate could be substituted for the amino acid mixture [2]. Various authors [2—5] agreed that the growth of *B. anthracis* was suboptimal in media containing only salts, amino acids and glucose. Growth was considerably "stimulated" by the addition of certain metabolites, especially of thiamine or purine bases.

The observed stimulating effect can not be, however, considered to represent the essential requirements of the bacteria. Therefore, we attempted to isolate *B. anthracis* mutants for which the vitamins or nucleic acid bases were not simple stimulants, but essential growth factors. In a preliminary paper we reported on various mutants isolated from *B. anthracis* [6]. One of us presented a detailed description of mutants which developed in unsupplemented amino acid medium at room temperature, but were unable to grow at 34° C or more without the addition of thymine or thymidine [7, 8].

The penicillin method recommended for the isolation of Enterobacteriaceae auxotrophs [9, 10] seemed inadequate for obtaining auxotrophs from the *Bacillus* species producing generally penicillinase. By its penicillin sensitivity, *B. anthracis* can well be distinguished from the intensively penicillinase producing *B. cereus* [11, 12], although *B. anthracis* may also produce slight amounts of penicillinase [13]. WACHSMAN and MANGOLO [14] recommended

8-azaguanine for the isolation of auxotrophs from penicillinase-producing organisms. The method was particularly suitable for obtaining *B. megaterium* auxotrophs, and has successfully been applied for the isolation of *B. subtilis* and *B. atterimus* auxotrophs (MARJAI, CSISZÁR and MOLNÁR, unpublished data). With a suitable modification, the azaguanine method yielded some auxotrophs from *B. anthracis*. More successful isolations were performed by the use of spores obtained after exposing bacterial suspensions to ethyl-methane sulphonate. This paper gives an account of these experiments and describes the properties of *B. anthracis* auxotrophs.

Materials and methods

Organism. The acapsulogenic mutant of *B. anthracis* strain Vollum was used. The mutant designated in our previous work as VC⁻ [15] displayed a slight tendency for reversion to the capsulogenic form. This property will be dealt with in a subsequent paper.

Chemicals. All substances were of analytical purity grade. Purine and pyrimidine bases were supplied by Fluka A. G. (Buchs). Agar obtained from the USSR was washed several times in distilled water prior to use. From the washed agar sterile 3 per cent gel was prepared.

Basal casein medium (BCM) supplemented with thiamine. The double strength medium contained the following ingredients. Vitamin-free Casamino acids (Difco), 25 g; L-glutamic acid, 3.5 g; DL-tryptophan and L-cystine, 0.125 g; sodium citrate, 2 g; K₂HPO₄, 10 g; Na₂SO₄, 0.5; MgSO₄ · 7 H₂O, 0.5 g; ferric ammonium citrate, 25 mg; MnSO₄ · 4 H₂O, 10 mg; thiamine 5 mg; distilled water, 2500 ml. The pH was adjusted to 7.2 with N NaOH. The solution was distributed and then sterilized at 110° C for 30 minutes. Prior to use the medium was diluted with an equal volume of distilled water or 3 per cent aqueous agar, and to each 100 ml aliquot 1 ml 20 per cent separately sterilized glucose solution was added.

Unless otherwise indicated, the basal casein medium was used with thiamine. The final concentration of this vitamin was 1 µg/ml.

Yeast extract peptone (YP) medium was prepared as described previously [16].

Homogeneous spore suspension. The method presented in reference 15 was used.

Isolation of auxotrophic mutants from cultures treated with 8-azaguanine was performed by the modified method of WACHSMAN and MANGOLO [14]. Logarithmic phase cultures of strain VC⁻ grown in YP medium were centrifuged and washed in saline. The washed suspension was irradiated with a low pressure mercury vapour U. V. lamp. The proportion of surviving cells was 10⁻³. The treated bacterial suspension was diluted 1:20 with YP medium. The tubes were gently shaken for 5–6 hours at 37° C. This incubation period allowed the segregation of mutants enclosed in bacterial chains and thus rendered them able to produce colonies consisting of homologous cells. After centrifugation and washing in saline, 1 part of deposit was resuspended in 100 parts of BCM solution. Of the suspension 2 ml was pipetted into 10 ml BCM medium. Three cultures were prepared in this manner and incubated until the increase in turbidity had become appreciable. Then 150 or 75 µg/ml 8-azaguanine was added and the cultures were reincubated. After 90, 120 and 160 minutes suitably diluted aliquots were inoculated onto YP plates.

Isolation of auxotrophs from cultures exposed to ethyl-methane sulphonate (EMS). VC⁻ spores were inoculated into liquid YP medium, then the logarithmic phase culture containing 2.5–3 × 10⁷/ml colony forming units, was centrifuged. The deposit was washed twice in pH 7.2, 0.04 M sodium phosphate buffer. The washed bacteria were resuspended in buffer to the original volume. After the addition of EMS at 0.3 M concentration and incubation in a 37° C water bath for 30 minutes, the suspension was cooled rapidly and centrifuged while cold. EMS treatment resulted in a 4 to 5 log units decrease in the colony former count. Subsequently the bacteria were washed twice and aliquots of a dense suspension were transferred into 20 ml YP medium. The cultures were shaken at 37° C until sporulation had occurred (80–90 hours). The culture was homogenized in order to give an adequate spore suspension for the isolation of auxotrophs.

Isolation of auxotrophs. Negative or sporulated bacteria exposed to the mutagenic effect were streaked onto YP agar plates so that on each plate 40–60 colonies were formed. Incuba-

tion lasted for 12–13 hours at 37°C. The young cultures consisting of colonies about 2 mm in diameter were replica plated by the velvetten stamp method [17] onto BCM agar. After comparison of the plates, 25–30 colonies, being apparently auxotrophic mutants, were transferred by needle point inoculation to YP plates. The developing young colonies were replica plated onto media containing a mixture of vitamins (folic acid, pyridoxine, pyridoxal, pantothenic acid, nicotinamide, p-amino benzoic acid, each 0.1 µg/ml, lactoflavin 0.25 µg/ml, biotin 0.01 µg/ml, cyanocobalamine 0.005 µg/ml) or a mixture of purine bases (hypoxanthine, xanthine, adenine, guanine) and pyrimidine bases (uracil, thymine, cytosine) each at 10 µg/ml concentration. The auxotrophs selected in preliminary tests were then examined for special requirements. After establishing the requirements, the strains were inoculated on YP agar slants and homogeneous spore suspensions were prepared. Final determination of requirements was carried out by use of these suspensions. Saline spore suspensions were used for storing the auxotrophs.

The assay of growth response. The quantitative requirements of some auxotrophs were estimated in liquid medium. A series of tubes containing graded amounts of the required metabolite (e.g. biotin) were set up. The volumes were made up to 2.5 ml with distilled water, then 3 ml double strength BCM solution was added to each tube. The tubes were closed with aluminium foil and sterilized in the autoclave. After inoculation with 0.1 ml spore suspension containing 100–200 spores, to each tube 0.4 ml sterile 3.3 per cent glucose solution was added. The tubes were incubated at a slanted position. Growth was expressed in optical density units measured at 600 mµ, or in the protein content of washed bacteria. The latter method was used when the bacteria had formed large aggregates in the culture. Protein was determined by MILLER's method, by FOLIN's reagent [18].

Measurement of the accumulation of aryl-amides. Purine deficient auxotrophs were inoculated into liquid BCM medium containing 10 µg/ml hypoxanthine. After incubating overnight at room temperature, the culture was diluted 1 : 10. Then various amounts of hypoxanthine ranging from 5 to 50 µg/ml were added to aliquots of the dilution. The cultures then were gently shaken at 37°C for 4–6 hours. After centrifugation the imidazol-amine content of the supernatant was determined by the method of RAVEL *et al* [19]. The degree of accumulation was estimated by measuring the optical density at 540 mµ in cells of 1 cm light path.

Results

Cultural properties of VC⁻ parent strain and its auxotrophs. On YP agar more or less circular, dull colonies 7–8 mm in diameter developed from the spores of the parent strain. On BCM agar smaller colonies were produced. When thiamine was omitted from the medium, convex colonies at most 1–1.5 mm in diameter developed. As to thiamin dependence, the spore suspension of the parent strain was apparently nonhomogeneous. On the medium devoid of thiamine partly small, very flat, partly somewhat larger colonies were formed. The colonies showed no such difference when grown on BCM agar supplied with 1 µg/ml of thiamine. On that medium 2–2.5 mm, uniform, high convex colonies developed (Fig. 1).

As one of us has reported [8], on casein hydrolysate medium containing purine and pyrimidine bases and various water-soluble vitamins, strain VC⁻ produced high convex colonies 5–6 mm in diameter. It has been pointed out that the nutrient requirements of strain VC⁻ were considerably influenced the incubation temperature. Between the range of room and body temperature, the minimal nutritional requirements of the strain increase with the increase of the temperature. The increase for thiamine requirement is particularly definite, and sufficient amounts of this substance effectively reduce the inhibitory effect of higher temperatures (Table I).

It is seen that in thiamineless BCM medium the degree of growth was greatly decreased by a rise in temperature. Addition of 1 $\mu\text{g}/\text{ml}$ thiamine not only enhanced growth, but also eliminated the inhibitory effect of higher temperatures. Thiamine therefore was considered an important additional factor in the basal medium.

On BCM agar the spores of all auxotrophs germinated. In some auxotrophic strains (e.g. in the riboflavin-requiring mutants) the spore developed into a vegetative form, which was unable to divide. In the majority of the

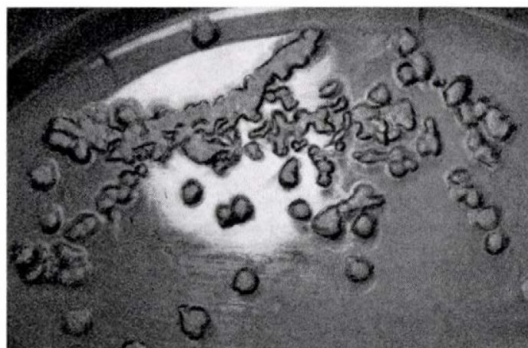


Fig. 1. Colonies of parent strain VC^- on BCM agar. (37°C , 24 hrs. Magnification $\times 1.5$)

Table I

Thiamine requirement of parent strain VC^- at different temperatures
(Optical density values in liquid medium after 72 hours' incubation)

Medium	Growth at different temperatures			
	26°	34°	37°	39°
BCM without thiamine	0.40	0.26	0.08	0.00
BCM with 1 $\mu\text{g}/\text{ml}$ thiamine	0.69	0.69	0.66	0.45

strains some divisions occurred after spore germination, and thus very small colonies consisting of small numbers of cells developed. The degree of growth was characteristic of each auxotrophic strain. The various types of this minimum multiplication, named abortive growth, are presented as follows. Micrographs 2a and 2b show abortive growth type 1. This type of growth was exhibited on BCM agar by most of the purine dependent auxotrophs. When 10^4 – 10^5 spores of these strains were inoculated onto one plate, germination and development to vegetative forms occurred, but no microcolonies were produced. The abortive growth of pyrimidine or vitamin deficient strains was more definite. This kind of growth, designated type 2, is shown in Fig. 3.

Some auxotrophs, mainly biotin-requiring strains, produced on BCM agar very effuse colonies larger than 1 mm in diameter. It was characteristic that the colonies reached a maximum size in 24 hours (Type 3 in Fig. 4).

Abortive growth was presumably due to contaminating substances present in the medium. It is probable that not the agar, but the casein hydrolysate contained these factors. Some Casamino acids lots were encountered, which induced a much more definite abortive growth than the forms described

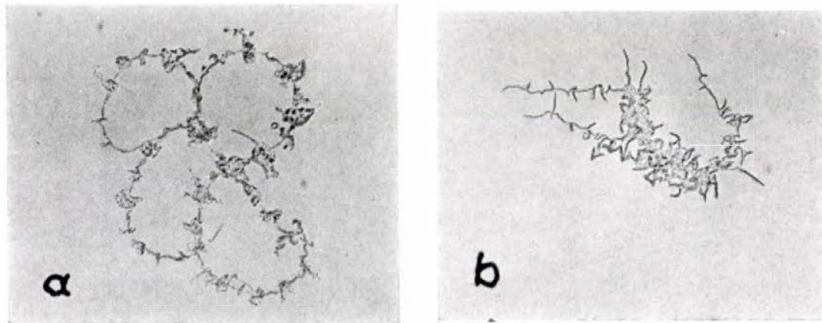


Fig. 2. Type 1 abortive growth on BCM agar (37° C, 24 hrs. Magnification $\times 56$). (a) Strain No. 58, (b) Strain No. 5. Both strains require purine (Pu 1)

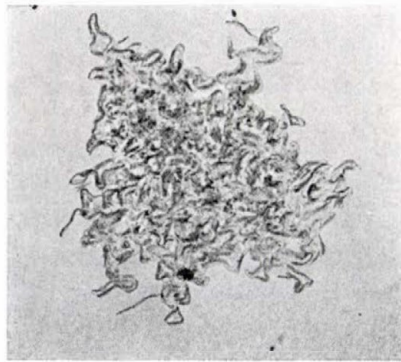


Fig. 3. Type 2 abortive growth (37° C, 24 hrs. Magnification $\times 56$). Nicotinamide dependent strain No. 18

above. These more contaminated lots were not used in further experiments. The contamination of Casamino acids is indicated also by the fact that mainly biotin deficient strains showed enhanced abortive growth (type 3). Riboflavin, which apparently was destroyed by acid hydrolysis of the casein, seemed to play no part as a contaminating substance. When BCM plates were inoculated with a heavy inoculum of spore material of the auxotrophs, this resulted in spore germination but no visible colony formation. This indicates

that the heavy inoculum had exhausted the contaminating substance accessible to the organisms.

The different degrees of abortive growth caused no difficulty in determining the requirements of the auxotrophs. On BCM medium supplemented with the corresponding vitamin or base, the auxotrophs formed dense, convex colonies 3–4 mm in diameter after an incubation for 24–48 hours. The colonies were almost identical with those of the parent strain grown on BCM agar.

Part of the auxotrophic mutants displayed a change in the appearance of their abortive colonies on prolonged incubation. Such strains developed

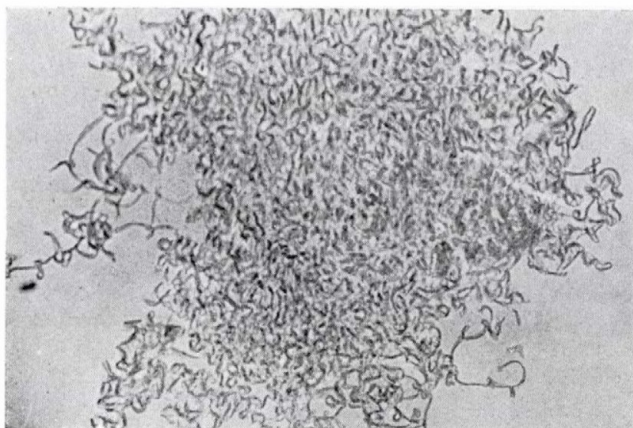


Fig. 4. Type 3 abortive growth (37° C, 24 hrs. Magnification $\times 56$). Biotin dependent strain No. 37

0.5–1 mm, or sometimes larger colonies after 48–72 hours but they usually did not reach the size attained in the presence of the adequate metabolite. These strains were imperfect auxotrophs, similar to the “leaky” mutants isolated from various species of bacteria.

A genotypic alteration in the isolated strains was indicated by the finding that the mutants had retained their specific nutritional requirements on serial subcultures. The change was therefore not of the phenotypic character. The population of the auxotrophs contained some revertants. No attempts were made to estimate the rate of back mutation more exactly, because chain formation presented considerable technical difficulties. Some rough estimates were performed on BCM agar with spore suspensions obtained from YP agar slant cultures started from one single auxotrophic colony. With various strains the proportion of revertants among the spores varied between 10^{-4} and $<10^{-6}$. The figure $<10^{-6}$ indicates that no revertants occurred in a culture consisting of several millions of spores. Of the 24 examined strains 11 yielded no back mutants under the above mentioned experimental conditions.

Efficiency of the isolation of auxotrophs. By means of the azaguanine method only a small number of auxotrophs were isolated. Examination on five different occasions of altogether 6013 colonies resulted in the isolation of 5 auxotrophs. Of these 3 strains were thymine and thymidine dependent at 37° C; at room temperature they grew without these factors. (The finding has been detailed by one of us in a separate paper [8].) Of the two remaining auxotrophs one required uracil, the other riboflavin.

The poor yield might partly be explained by the fact that the isolation procedure was started with vegetative forms. In case of vegetative bacteria the individual colony former, that is, the bacterial chain may consist of mutants beside prototrophic cells. The segregation of auxotrophic mutants might be insufficient during cultivation and thus the colonies appear as prototrophs.

Table II

Auxotrophs isolated with the ethyl-methane sulphonate technique

Experiment	No. of colonies examined	Requirements of the auxotrophs				Total	%
		purine	pyrimidine	vitamin	?		
1	350	6				6	1.71
2	1548	9		3		12	0.78
3	2437	9	4	4	4	21	0.86
4	4914	75	49	17	1	143	2.90

? = Requirements not defined.

The EMS method exerted a much more intensive mutagenic action. This technique was advantageous because, instead of vegetative forms, it allowed the use of homogeneous spore suspensions for the screening of auxotrophs. During the course of these investigations 9249 individual colonies were tested in four different experiments; 182 of them were found to be auxotrophs. The results of these investigations are summarized in Table II.

It may be seen that the isolation frequency of the auxotrophs varied with different experiments. The distribution according to requirements was also variable.

The results obtained with these 182 mutants, and with 5 strains isolated with the azaguanine technique are described as follows.

Purine auxotrophs. With some exceptions, on BCM medium these mutants produced type 1 abortive growth. In order to demonstrate their requirements, spore suspensions were inoculated on BCM agar plates supplemented with 10 µg/ml adenine, guanine, hypoxanthine or xanthine. The results were read after 24, 48 and sometimes 72 hours incubation at 37° C; parallel cultures were incubated at 26° C. All 99 purine auxotrophs required the corresponding purine

derivative at both temperatures. Among the isolated auxotrophs "leaky" mutants were encountered. With these some growth was observed on the minimal medium after 48 hours; on further incubation the colonies sometimes became larger. The mutants were classified into groups according to their purine base utilization pattern (Table III).

The 99 purine auxotrophs were thus divided into 6 groups. Most strains (Pu 1) utilized all four bases, that is, the block was prior to the hypoxanthine nucleotide. These phenotypically uniform strains differed in their genotype. Only some strains of Pu 1 were, however, studied to determine the place of the

Table III

Distribution of purine auxotrophs according to utilization of various purines

Group	Utilization of				No. of strains	No. of leaky strains
	HX	Ad	X	Gu		
Pu 1	+	+	+	+	77	20
Pu 2	—	—	+	+	1	0
Pu 3	—	—	—	+	12	3
Pu 4	—	+	—	—	4	0
Pu 5	+	—	+	+	3	0
Pu 6	+	—	+	—	2	0

block in their purine pathway. Of 12 strains selected at random, one accumulated a diazotable precursor; the other strains gave the test with a negative result. In the former strain the block could be supposed either after 5-amino-4-imidazole-carboxamide ribotide or after the aminoimidazole ribotide. In the other mutants the formation of the imidazole ring, or the biochemical process following the 5-formamido-4-imidazole carboxamide ribotide was blocked.

Almost unexceptionally, hypoxanthine was best utilized by Pu 1 strains. Inosine was likewise well utilized. Part of the strains grew less rapidly on xanthine or guanine containing media than in the presence of hypoxanthine. This was apparent from the size and compactness of 24 hour colonies. Of the purines, adenine was generally less readily utilized. In the presence of this base an initial growth was observed after 24 hours only. Apart from some strains, further incubation resulted in a growth equal to that found in the presence of the other purines.

In mutants belonging to groups Pu 2 and Pu 3, guanine synthesis is lacking. Pu 2 strains are incapable of oxidizing inosinic acid to xanthylic acid. Pu 3 mutants are deficient in the amination of xanthylic acid. In Pu 4 strains capable of utilizing only adenine, the conversion of hypoxanthine into adenine or, more exactly, that of inosinic acid into adenylic acid, is blocked. Two

enzymes are known to be involved in this conversion. One of them splits off fumaric acid from adenylysuccinic acid. The same enzyme plays a part in the early stage of purine synthesis [20].

Mutants belonging to group Pu 5 did not utilize adenine. The reason for this has not been studied.

The adenine and guanine deficiency in Pu 6 mutants cannot be explained without further investigations.

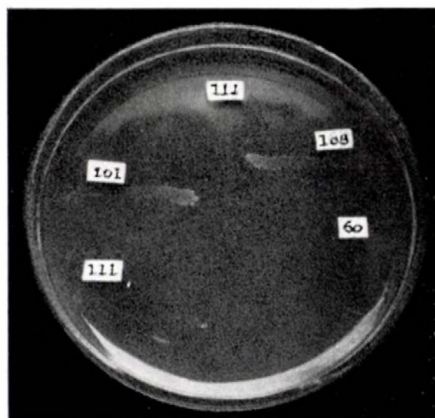


Fig. 5. Syntropism between individual uracil dependent strains. BCM agar, 37° C, 28 hours

Pyrimidine auxotrophs. Of the 57 pyrimidine dependent strains 49 utilized only uracil. For these mutants uracil could not be replaced by other pyrimidine bases (cystosine, thymine). Of the 49 mutants 7 were found to be leaky ones. They slowly formed small colonies at both 37° C and 26° C, even in the absence of uracil. Nevertheless, without uracil neither of these strains developed well at 26° C. Strains, the requirement of which is met by uracil, may differ in their genotype, for between certain strains a syntropism could be revealed (Fig. 5). This means that different intermediates accumulate in individual strains. The growth response of a uracil auxotroph is shown in Fig. 6.

One of the thymine dependent mutants has been described and studied in detail [8]. The thymine auxotrophs isolated were found capable of utilizing both thymine and thymidine-5-phosphate. However, four strains of them grew poorly even at very high concentrations of thymine, while the others readily utilized this base. In the presence of thymidine all mutants exhibited a good growth.

It was characteristic of the thymine auxotrophs that they needed thymine only at temperatures higher than 32° C. Except one strain (No. 194) they grew well at room temperature on pyrimidineless medium. Strain 194 required thymine at room temperature as well.

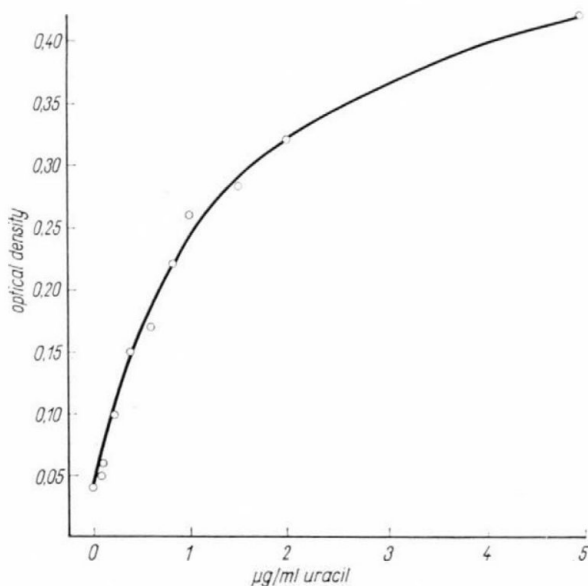


Fig. 6. Growth response of uracil dependent strain No. 12. Liquid BCM cultures, 37° C, 24 hrs. The growth response at different uracil concentrations is expressed in optical density values

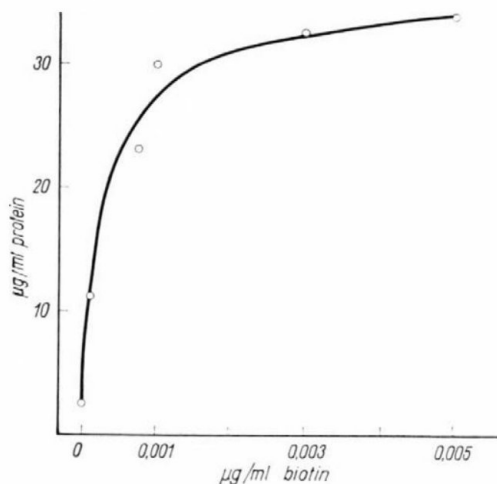


Fig. 7. Growth response of biotin dependent mutant No. 37. Liquid BCM culture, 37° C, 24 hrs. Growth at different biotin concentrations is expressed by the protein content of bacteria

Vitamin auxotrophs. Altogether 25 vitamin deficient strains were isolated. Of these 20 were biotin auxotrophs. It is remarkable that out of the 20 mutants 10 required this vitamin only at 37° C; at 26° C these mutants multiplied freely. The growth response at 37° C of one of these strains is presented in Fig. 7.

Among 5 further isolated mutant 2 required nicotinamide, 2 PABA and 1 riboflavin for their growth. These mutants needed the vitamins both at 37° C and 26° C.

The requirements of 5 auxotrophic strains isolated during the course of these investigations could not be defined exactly. As neither of these strains grew in the presence of the bases, nucleosides, and vitamins used in the experiments, no further attempts were made to determine their requirements.

Discussion

The acapsulogenic mutant of *Bacillus anthracis*, strain Vollum yielded a considerable number of mutants which, as compared to the parent strain, exhibited increased nutritional requirements. The requirements for certain purine and pyrimidine bases and vitamins were essential. Purine dependent auxotrophs were encountered most frequently. Species belonging to Enterobacteriaceae synthesize purine bases *de novo*. A great number of auxotrophic mutants requiring purine bases for growth were isolated and studied from these species. According to hypoxanthine, xanthine, guanine and adenine utilization, MAGASANIK [21] classified purine mutants of *E. coli*, *S. typhimurium* and *A. aerogenes* into four groups. Group 1 included mutants growing in the presence of any of these bases. Members of group 2 utilized only xanthine and guanine but not hypoxanthine or adenine. Group 3 grew only with guanine and group 4 only with adenine.

Considering the utilization pattern, the purine mutants of *B. anthracis* were divided into 6 groups. Groups Pu 1, Pu 2, Pu 4 corresponded to the above described classification of Enterobacteriaceae mutants. The extraordinary behaviour of Pu 5 and Pu 6 strains could not be explained on the basis of known data. Pu 5 did not utilize adenine even after prolonged incubation. Two strains belonging to group Pu 6 utilized oxy-bases but not amino derivatives (adenine and guanine). The lack of utilization of amino-purines might be due to permeability conditions or other reasons (*e.g.* feed-back inhibition).

While no typical thermosensitive mutants (dependent only at 37° C) occurred among the purine auxotrophs, they constituted a considerable part of pyrimidine and vitamin dependent strains. Under the experimental conditions applied, the thermosensitive mutants required additional metabolites at 37° C; at 26° C their nutritional requirements were identical with those of the parent strain. Thermosensitive mutants of *E. coli* were first isolated by DAVIS and MAAS [22]. These strains required pantothenic acid at temperatures higher than 32° C. This property was associated with the extreme heat-sensitivity of pantothenate-synthetase. Similar mutants have been isolated

from other micro-organisms. Investigations concerning this issue have been excellently reviewed by FINCHAM [23].

The auxotrophic mutants of *B. anthracis* are interesting from two points. On the one hand they may be useful in studies on the recombination of genetic markers, and, on the other, investigations of these mutants may yield some idea as to the biochemical pathways playing a role in the virulence of this pathogen. Our experiments concerning this problem are in progress.

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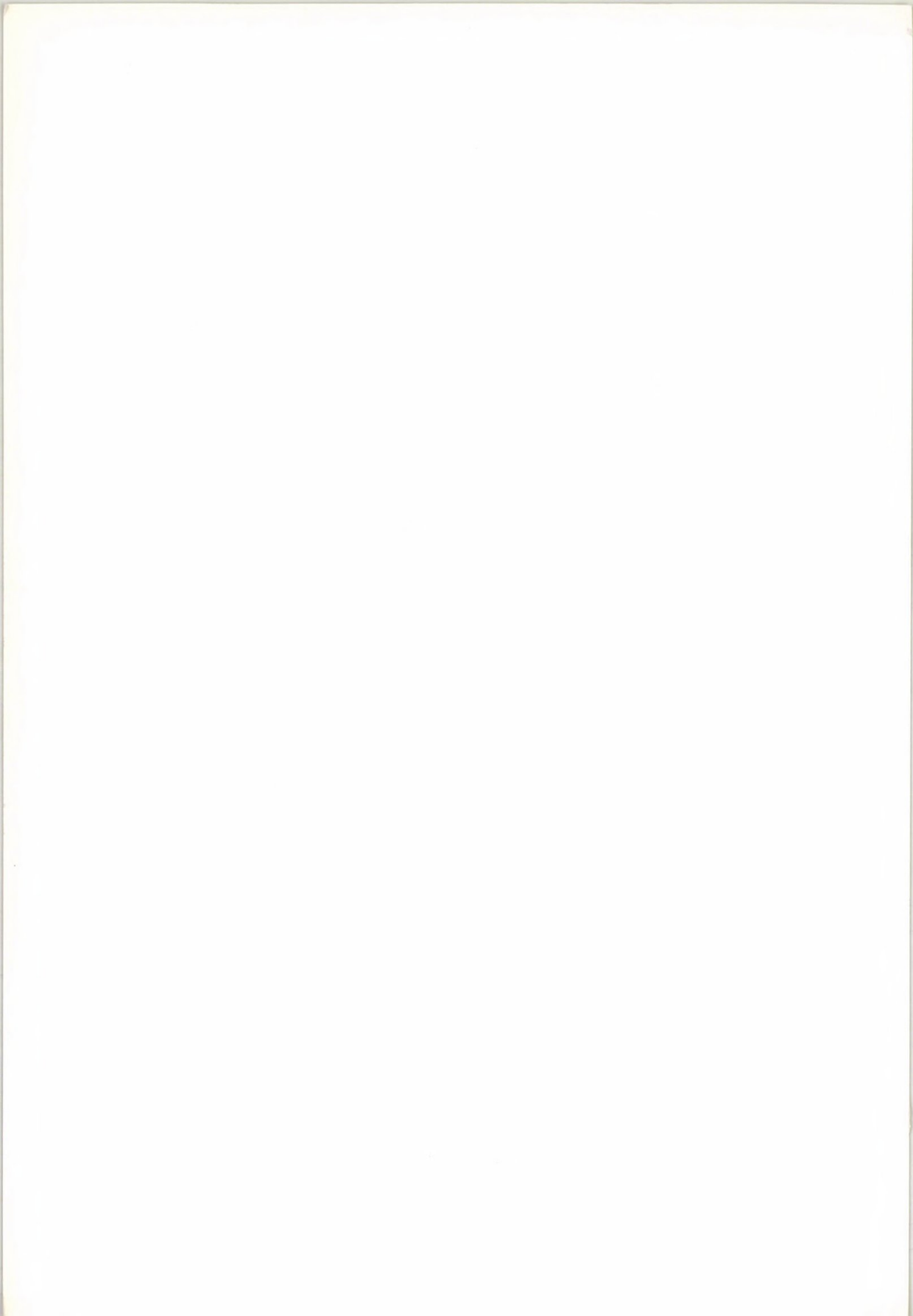
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ТОМ X

РЕЗЮМЕ

P. Geck, P. Osváth, B. Voltay, R. Backhausz, G. Losonczy, G. Vigh, S. Bognár :

IMMUNOFLUORESCENCE AND PASSIVE HAEMAGGLUTINATION IN INFANTILE ENTEROCOLITIS

ИССЛЕДОВАНИЯ МЕТОДАМИ ИММУНОФЛЮОРЕСЦЕНЦИИ И ПАССИВНОЙ ГЕМАГГЛЮТИНАЦИИ ПРИ ДЕТСКИХ ЭНТЕРОКОЛИТАХ

П. Гекк, П. Ошват, Б. Волтай, Р. Бакхаус, Г. Лошонци, Г. Виг, Ш. Богнар

Исследовалось выделение шигелл у 252 детей находящихся на лечении в больнице с энтероколитом. Возраст детей от 1 года до 14 лет. Исследовалось как и содержимое прямой кишки культивированием бацилл, методом иммунофлюоресценции и пассивной гемагглютинации. Из содержимого прямой кишки, взятого при поступлении детей в больницу, изолированы шигеллы в 40,5% случаев, из кала полученного на следующий день только 12,7%. В 187 случаях результаты культивирования сопоставлялись с данными, полученными методом иммунофлюоресценции. Результаты, полученные последним методом, совпали в 89,4% случаев с данными культивирования, в то же время методом иммунофлюоресценции получили в 33 случаях положительные результаты в кале, давшем с обоими методами культивирования отрицательный результат. Сочетанием этих трех методов удалось выявить шигеллы в 53% случаев. Повышения титра, полученные при двукратном исследовании сывороток 18 больных методом пассивной гемагглютинации, подтвердили обнаруженные прежними методами положительные результаты. Повышение титра наблюдалось иногда даже у больных, у которых никаким другим методом выявить шигеллы не удавалось.

E. K. Novák :

OLIGOSACCHARIDE DECOMPOSITION BY CANDIDA SOLANI

ФЕРМЕНТАТИВНАЯ СПОСОБНОСТЬ CANDIDA SOLANI В ОТНОШЕНИИ ОЛИГОСАХАРИДЫ

E. K. Новак

При изучении ферментативной способности *Candida solani* установлено, что сахара роза разлагается внутриклеточно на глюкозу и левулезу, но разложение не производится инвертазой или мальтазой (α -глюкозидазой). Разложение мальтазы происходит также внутриклеточно, но не под влиянием α -глюкозидазы. Соответственно этому, *Candida solani* не содержит ни инвертазы, ни мальтазы. В пермеации мальтазы принимают участие анаэробный и аэробный пермеазный фермент. Анаэробная мальтозопермеаза является ферментом адаптивного характера.

B. Serény :

BIOCHEMICAL REACTIONS AND VIRULENCE OF *E. COLI* 0124 : K72 (B17)

БИОХИМИЧЕСКИЕ РЕАКЦИИ И ВИРУЛЕНТНОСТЬ *E. COLI* 0124 : K72 (B17)

Б. Шерень

Несмотря на то, что некоторые биохимические реакции *E. coli* 0124 и шигелл совпадают, все таки по совокупности характерных биохимических свойств *E. coli* 0124 принадлежит к группе штаммов *E. coli*, которые медленно разлагают лактозу. Проводя исследования при косоугольном освещении, в культурах *E. coli* 0124 можно распознать различные морфологические варианты колоний, которые могут расходиться в вирулентности, у морских свинок, зараженных конъюнктивально вирулентными культурами *E. coli* 0124, наблюдается типичный кератоконъюнктивит. В отношении природного приобретенного иммунитета штаммы шигеллы и *E. coli* 0124 взаимно заместимы, создается перекрестный иммунитет.

M. Fodor, F. Rozgonyi, E. Csépe :

CORRELATION BETWEEN PHAGE-TYPE, COAGULASE, HYALURONIDASE AND PHOSPHATASE ACTIVITY, AND MERCURIC CHLORIDE RESISTANCE OF STAPHYLOCOCCUS AUREUS

ВЗАИМОСВЯЗЬ МЕЖДУ ТИПОМ ФАГА, АКТИВНОСТЬЮ КОАГУЛАЗЫ, ГИАЛУРОНИДАЗЫ, ФОСФАТАЗЫ И РЕЗИСТЕНТНОСТЬЮ К СУЛЕМЕ ШТАММОВ STAPHYLOCOCCUS AUREUS

М. Фодор, Ф. Розгони, Е. Чепке

Штаммы *Staphylococcus aureus*, принадлежащие к первой группе фагов и в первую очередь штаммы с типом фага 80/81, обладают низкой активностью коагулазы и гиалуронидазы и высокой активностью фосфатазы. Штаммы второй группы фазов показывают высокую активность коагулазы и гиалуронидазы, но среднюю степень активности фосфатазы. Штаммы третьей группы фагов имеют крайне низкую активность фосфатазы, среднюю степень активности гиалуронидазы и коагулазы. Большинство так называемых «эпидемических штаммов» являются резистентными к сулеме, но последнее свойство не имеет взаимосвязи с повышенным образованием ни одного из исследованных энзимов.

E. Szép, S. Szabó :

ADAPTATION OF YEASTS TO DISINFECTANTS

АДАПТАЦИЯ ДРОЖЖЕЙ К ДЕЗИНФИЦИРУЮЩИМ СРЕДСТВАМ

Е. Сен, Ш. Кабо

Применяемые в производстве спирта 2 вида дрожжей *Saccharomyces cerevisiae* адаптировали в течение длительного времени к формалину, активному хлору, стерогену и пентахлорфенолату натрия. Адаптация производилась исходя из более низких концентраций дезинфицирующего средства. По отношению к начальной концентрации получили с формалином трехкратную, с активным хлором десятикратную, стерогеном трехкратную и с пентахлорфенолатом натрия пятикратную адаптацию. Эффективность адаптации проверялась ферментативными пробами с положительным результатом. Адаптированные дрожжи давали брожение на питательных средах с такой концентрацией дезинфицирующего средства, которая не способствовала росту не адаптированных дрожжей. На питательных средах, содержащих дезинфицирующее средство, затягивается первая (lag) фаза размножения, но в логарифмической фазе скорости брожения, а также количество спирта, дрожжей и остаточного сахара к концу ферментации были одинаковыми с контрольными.

I. Nász, G. Kulcsár, P. Dán, A. Lengyel, I. Cserba :

STUDIES ON THE AETIOLOGY OF EPIDEMIC KERATOCONJUNCTIVITIS
OBSERVED IN BUDAPEST DURING WINTER, 1961—1962

ИЗУЧЕНИЕ ЭТИОЛОГИИ ЭПИДЕМИИ КЕРАТОКОНЬЮНКТИВИТА
ПРОТЕКАВШЕЙ ЗИМОЙ 1961—1962 ГГ. В БУДАПЕШТЕ

И. Нас, Г. Кулчар, П. Дан, А. Лендель, И. Черба

Из промывной жидкости конъюнктивы 58 больных на тканевой культуре HeLa и Detroit-6 выделили 9 штаммов вируса, которые на основе реакции связывания комплемента оказались аденовирусами. С помощью реакции задержки гемагглютинации и нейтрализации вируса смогли включить 7 из них в восьмой тип и 2 в шестой тип. Серологическими пробами двукратно исследовали 38 сывороток от тех-же лиц, у которых брали материал для культивирования. Пробами связывания комплемента и нейтрализации обнаружилось повышение титра антител аденовируса типа 8 в 78% случаев. Реакцией задержки гемагглютинации выявилось повышение титра в 94% случаев, полученные титры в последнем случае были значительно выше, чем наблюдаемые при первых двух реакциях. На основании исследований главным этиологическим фактором эпидемии можно считать аденовирус типа 8.

H. Milch, V. G. László, G. Biró :

SUPPLEMENTED PHAGE-TYPING OF SALMONELLA TYPHI MURIUM
AND ITS USE IN EPIDEMIOLOGY

ДОПОЛНЕНИЕ К ОПРЕДЕЛЕНИЮ ФАГОВОГО ТИПА ШТАММОВ SALMONELLA
TYPHI MURIUM И ЭПИДЕМИОЛОГИЧЕСКОЕ ПРИМЕНЕНИЕ МЕТОДА

Х. Мильх, В. Г. Ласло, Г. Биро

Исследовался фаговый тип 1895 штаммов *Salmonella typhi murium* с помощью типовых фагов по Felix—Callow. Этот метод, из-за частой встречаемости отдельных фаговых типов и распространенности атипичных, не типизируемых или деградированных штаммов, является неудовлетворительным для эпидемиологических целей.

При дополнении метода осваивали ферментационную типизацию Kristensen, по видоизменной методике Kallings, а проводили исследования по лизогенности и определяли свойство выявленных темперируемых фагов.

Самыми частыми фаговыми типами по схеме Felix—Callow явились (по очагам): 2b, 4,5, 1a, 1a var. 1, но встречались и типы 1,1b, 2,2a, 2c, 3 и другие не включаемые в схему самостоятельные типы. Лизогенные свойства имели 76,6% исследованных штаммов.

На основании свойств (литическая активность, термочувствительность) темперируемого фага различных штаммов, внутри отдельных фаговых типов стало возможным дальнейшее распределение. Сопоставляя результаты полученных при распределении типов с эпидемиологическими данными установлено, что непосредственное определение фагового типа, дополненное темперирующей фаг-идентификацией и ферментационной типизацией для эпидемиологических целей хорошо применимо.

I. Nász, P. Dán, G. Kulcsár, A. Lengyel, I. Cserba :

ACCIDENTAL LABORATORY INFECTIONS BY ADENOVIRUS TYPE 8

ЛАБОРАТОРНОЕ ЗАРАЖЕНИЕ АДЕНОВИРУСОМ ТИПА 8

*И. Нас, П. Дан, Г. Кулчар, А. Лендель,
И. Черба*

При изучении этиологии эпидемии эпидемического кератоконъюнктивита, протекавшей в Будапеште зимой 1961—62 г. произошло лабораторное заражение 3 сотрудников выделенным вирусом типа 8. Во всех трех случаях развился типичный эпидемический кератоконъюнктивит. Вирус был выделен вновь только у двух больных, у третьего больного, которому спустя три часа после заражения начали проводить лечение колларголом, культивирование дало отрицательный результат. Титр специфических антител задерживающих гемагглютинацию и нейтрализующих вирус, в крови всех трех больных был повышен. У остальных сотрудников работающих с вирусом и их членов семьи не удалось выделить вирус и обнаружить в крови специфические антитела. Обсуждаются сделанные выводы по этиологии, распространению и некоторым эпидемиологическим свойствам эпидемического кератоконъюнктивита.

I. Béládi, Á. Kahán, E. Kukán, I. Mucsi, I. Pápai :

AETIOLOGIC RELATIONSHIP OF ADENOVIRUS TYPE 8 TO THE EPIDEMIC OF KERATOCONJUNCTIVITIS IN SZEGED

ЭТИОЛОГИЧЕСКАЯ РОЛЬ АДЕНОВИРУСА ТИПА 8 В ЭПИДЕМИИ ЭПИДЕМИЧЕСКОГО КЕРАТОКОНЪЮНКТИВИТА В ГОРОДЕ СЕГЕД

И. Белади, А. Кахан, Е. Кукан, И. Мучи, И. Папай

Во время эпидемии эпидемического кератоконъюнктивита, протекавшей в городе Сегед весной 1962 г. охватившей приблизительно 1500 лиц, произвели выделение вируса у 52 больных с типичной картиной заболевания. В тканевых культурах He La из промывной жидкости конъюнктивы у 13 больных удалось выделить аденовирус типа 8. Исследовалась двукратно сыворотка у 10 больных, у которых культивирование дало положительных результат. У 7 больных обнаружилось 4-кратное или более выраженное повышение титра вируснейтрализующих антител в отношении аденовируса типа 8. Из 15 больных, у которых выделение вируса не удалось, у 12 отмечалось повышение титра против того-же типа.

L. Vámos :

METABOLIC ROLE OF OLIGOSACCHARIDES IN ALCOHOLIC FERMENTATION BY YEASTS

РОЛЬ ОЛИГОСАХАРИДОВ В ОБМЕНЕ ДРОЖЖЕЙ ПРИ УСЛОВИЯХ СПИРТОВОГО БРОЖЕНИЯ

Л. Вамош

Роль олигосахаридов, возникающих при кислотном гидролизе крахмала, в анаэробном обмене дрожжей зависит от вида и варианта их. Исследованный штамм пекарских дрожжей может только утилизировать моно- и дисахариды, *Saccharomyces sake* трисахарид, *Schizosaccharomyces pombe* только олигосахариды с большими молекулами. На питательной среде, содержащей гидролизат крахмала и инокулированной *Schizosaccharomyces pombe* при брожении появляются два новых, отсутствующих в исходной среде, олигосахарида. На основании исследований автор указывает на возможный механизм возникновения этих двух олигосахаридов и на то, что сбраживание ди-, три и тетра-сахаридов питательной среды не осуществляется непосредственно, а путем межклеточного ресинтеза.

L. Váci, L. Géder, M. Koller, E. Jeney :

INFLUENCE OF TEMPERATURE ON THE MULTIPLICATION OF VARICELLA VIRUS

ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА РАЗМНОЖЕНИЕ ВИРУСА В ВЕТРЯНОЙ ОСПЫ

Л. Вацц, Л. Гедер, М. Коллер, Е. Йенеи

Размножение вируса ветряной оспы исследовалось на человеческих фибробластных тканевых культурах, которые содержались при температуре 30, 37, 39 °С. Размножение человеческих фибробластов и развитие однослойных культур происходит приблизительно одинаково при температуре 37 и 39 °С, но процесс значительно замедляется при температуре 30 °С. Для размножения вируса ветряной оспы температура 37 °С наиболее благоприятна, при температуре 30 °С синтез вируса сильно замедляется, а при температуре 39 °С репродукция совершенно прекращается. При температуре 39 °С синтез вируса задерживается в первоначальной (внутриядерной) фазе. Внутриклеточный вирус при этой температуре сохраняет свою заразительность по крайней мере в течение 7 дней. Замедленное размножение вируса при температуре 30 °С вероятно является результатом пониженного обмена веществ клеток.

A. Koch :

EFFECT OF TEMPERATURE ON THE POTENTIOMETRIC TITRATION CURVE OF INFLUENZA VIRUS

ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА ПОТЕНЦИОМЕТРИЧЕСКУЮ КРИВУЮ ТИТРОВАНИЯ ВИРУСА ГРИППА

A. Kox

Анализ потенциометрических кривых титрования одного грамма азота вирусного белка, полученных при различных температурах, выявил, что ионизирующие группы вируса — на основании кажущихся ионизирующих температур (Q') — можно разделить на три класса.

3-ий класс состоит из однородных групп, средняя величина Q' которых + 10 300 калорий, а ионизационная константа (pK') у них 9,75. Эти группы по всей вероятности являются остатками лизина- $\epsilon-NH_2$.

Средняя величина Q' во второй группе + 6400 калорий и pK' 6,97, соответственно этому эти группы весьма вероятно являются имидазольными-остатками гистидина.

Все остальные группы — находящиеся в кислой части переходящей зоны, а также группы первого класса — располагают величинами между + 2000 и + 3000 калорий, и таким образом их можно считать карбоксилами.

Методом термического анализа в диапазоне от pH 3,00 до pH 10,75 получены следующие значения pK' :

$pK' = 3,50$, $pK'_2 = 4,50$, $pK'_3 = 5,11$ (5,125); $pK'_4 = 5,73$, $pK'_5 = 6,97$ и $pK'_6 = 9,75$

Значения связывания кислоты и щелочи в отдельных группах хорошо совместимы с предварительно обнаруженными величинами.

Ионизация у терминальных амидных и карбоксильных групп не обнаружена.

T. Szent-Iványi :

STUDIES ON SWINE ENTEROVIRUSES

I. ISOLATION AND SEROLOGICAL GROUPING OF STRAINS

ИССЛЕДОВАНИЕ ЭНТЕРОВИРУСОВ СВИНЕЙ.

I. ВИРУСНЫЕ ШТАММЫ И ИХ СЕРОЛОГИЧЕСКОЕ ГРУППИРОВАНИЕ

T. Сент-Ивани

Выделяли вирус из кала 586 свиней различного возраста, из 36 свиноводств. Выделено всего 152 (26,7%) штамма энтеровирусов, резистентных к хлороформу, с цитопатогенным действием на тканевых культурах почки свиней. Из 41 отобранного, бляшечным способом очищенного штамма, смогли 36 с помощью иммунных сывороток, приготовленных с 20 штаммами, включить в 13 серологические группы. 5 штаммов не дали реакции нейтрализации ни с одной из приготовленных сывороток. Одна из этих групп состояла из штаммов, дающих перекрестные реакции с вирусами *Teschen*, *Talfan-disease*. Из 11 исследованных заграничных вирусных штамма ЕС SO нейтрализовались только штаммы *Sibalin SI80/4*, *Betts—T80*, *T52A*, а также *Riems* 52 иммунными сыворотками, приготовленными из отечественных штаммов.

T. Szent-Iványi :

STUDIES ON SWINE ENTEROVIRUSES

II. MORPHOLOGY OF THE CYTOPATHIC CHANGES PRODUCED IN SWINE-KIDNEY MONOLAYERS

ИССЛЕДОВАНИЕ ЭНТЕРОВИРУСОВ СВИНЕЙ, II. МОРФОЛОГИЯ ЦИТОПАТИЧЕСКИХ ИЗМЕНЕНИЙ, ВЫЗЫВАЕМЫХ ШТАММАМИ ВИРУСА В ОДНОСЛОЙНЫХ ТКАНЕВЫХ КУЛЬТУРАХ СВИНОЙ ПОЧКИ

T. Сент-Ивани

Исследована морфологическая картина цитопатологических изменений, вызываемых 41 местными и 11 полученными из заграницы энтеровирусными штаммами свиней на тканевых культурах свиной почки: помещая культуры в пробирки с коллодием и вынимая их, применялись различные методы гистологической окраски.

На основе морфологической картины вызываемых штаммами цитопатологических изменений, штаммы разделялись на две группы. Штаммы одной группы вызывали 1-ый тип изменений, который характеризуется групповым округлением клеток, образованием морфологически распознаваемой клеточной оболочки, ректическим распадом клеточных ядер (*karyorrhexis*) и появлением светлого участка около пикнотического ядра клеток. Вторая группа штаммов вызывала морфологические изменения, обозначаемые II типом, которые появлялись рассеянно в отдельных клетках, с глыбчатым распадом цитоплазмы и наличием ацидофильных образований подобных включениям цитоплазмы. В дальнейшем цитоплазматические глыбки располагались около пикнотического ядра клетки в виде неправильных отростков. Характер цитопатологических изменений, вызванных штаммами и является постоянным и характерным свойством штамма и включаемые на основании реакции нейтрализации вируса в общую серологическую группу штаммы всегда обуславливают те же самые цитопатологические изменения.

J. Zsolt, M. Perki, E. K. Novák :

TAXONOMIC STUDIES ON *PROCANDIDA ALBICANS*
I. FERMENTATION OF SUGARS

ТАКСОНОМИЧЕСКИЕ ИССЛЕДОВАНИЯ *PROCANDIDA ALBICANS*
I. ИССЛЕДОВАНИЕ САХАРНОГО БРОЖЕНИЯ

Й. Жолт, М. Перки, Е. К. Новак

Исследовалось сахарное брожение 200 штаммов дрожжевого гриба *Procandida albicans* (syn: *Candida albicans*) в отношении 6 диагностических сахаров. Установлено, что все штаммы быстро сбраживают глюкозу и мальтозу и не действуют на лактозу и раффинозу. В отношении сбраживания галактозы и сахарозы штаммы не ведут себя одинаково, даже при повторных, т. е. параллельных опытах давали и отдельные штаммы изменяющиеся результаты, хотя в случае 30 дневной инкубации обнаружили 84 т. е. 75% положительность. Соблюдая то, что стандартные в настоящее время методы идентификации всегда дадут этот же результат, предлагают для характеристики сахарного сбраживания *Procandida albicans* буквенное обозначение *dgsm* (буквы обозначают броженные сахара, в порядке: декстроза, галактоза, сахароза и мальтоза), где подчеркиванием соответствующих букв обозначается стабильность данного свойства.

E. K. Novák, J. Zsolt :

TAXONOMIC STUDIES ON *PROCANDIDA ALBICANS*
II. DISACCHARIDE-SPLITTING ENZYMES

ТАКСОНОМИЧЕСКИЕ ИССЛЕДОВАНИЯ *PROCANDIDA ALBICANS*
II. ИССЛЕДОВАНИЕ ЭНЗИМОВ, РАЗЛАГАЮЩИХ ДИСАХАРИДЫ

Е. К. Новак, Й. Жолт

Установлено, что исследованные 4 штамма *Procandida (Candida) albicans* внутриклеточно разлагают мальтозу и сахарозу. Клетки не содержат инвертазы и соответственно этому ни в живом, ни в гомогенизированном состоянии, ни в обработанном ацетоном виде не разлагают раффинозу. На основании исследований предполагается, что сахароза и мальтоза не разлагаются тем-же самым энзимом и, что разлагающий мальтозу энзим не может быть у дрожжей, известная и являющаяся в отношении сахарозы активной-мальтазы.

L. Géder, M. Koller, É. Gönczöl, E. Jeney, I. Gönczöl :

ISOLATION OF HERPES ZOSTER VIRUS STRAINS

ВЫДЕЛЕНИЕ ШТАММОВ ВИРУСА ПРИ HERPES ZOSTER

Л. Гедер, М. Коллер, Е. Гёнциёл, Е. Йенеи, И. Гёнциёл

Из содержимого пузырьков 8 больных с *Herpes zoster* выделены 6 штаммов вируса. Вирусные штаммы на основании характерных цитопатогенных изменений, нативно обнаруженных на человеческих фибробластных культурах, на основе эозинофильных внутриклеточных включений и связанности инфекции к клетке, а также на основе проб нейтрализации, проведенных с сыворотками больных в остром и реконвалесцентном периоде заболевания и внутриклеточного выявления вируса методом иммунофлюоресценции, оказались штаммами вируса *Herpes zoster*.

I. Jókay:

QUANTITATIVE PRECIPITATION
AND ENZYME INHIBITION BY ANTIPHOSPHORYLASE

КОЛИЧЕСТВЕННАЯ ПРЕЦИПИТАЦИЯ И ЗАДЕРЖИВАЮЩЕЕ ВЛИЯНИЕ
АНТИФОСФОРИЛАЗНЫХ СЫВОРОТОК НА ЭНЗИМ

И. Йокаи

Проводилась количественная преципитация с кристаллической мышечной фосфо-рилазой-б кролика и гомологичными сыворотками петуха, регистрировалась задержка энзимной активности. Установлено, что задерживающее влияние сыворотки на энзим пропорционально содержанию в ней антител, если относительная задержка не достигает 66%. Задерживающее влияние сыворотки на энзим выражалось в антифосфоорилазных единицах. При преобладании антигена тождественное количество антисыворотки может вдвое больше задерживать активность фосфоорилазы, чем при точке эквивалентности. Между задерживающими величинами и содержанием преципитирующих антител сыворотки существует только примерная пропорциональность. Автор анализирует причины дан-ного явления.

I. Jókay, A. Kiss:

PLASMA PHOSPHATASE, CHOLINESTERASE
AND PEPTONASE IN ANAPHYLACTIC SHOCK

АКТИВНОСТЬ ФОСФАТАЗЫ, ХОЛИНЭСТЕРАЗЫ И ПЕПТОНАЗЫ ПЛАЗМЫ
ПРИ АНАФИЛАКТИЧЕСКОМ ШОКЕ

И. Йокаи, А. Киши

При анафилактическом шоке активность фосфатазы плазмы у морских свинок повышается до 111%, у кроликов 54,5—63%. Обнаружено 30% снижение холинэстеразы плазмы, в то время как активность пептоназы плазмы у кроликов повышалась на 17%.

M. Koller, É. Gönczöl, L. Vácz:

STUDY OF THE MULTIPLICATION OF VARICELLA-ZOSTER VIRUS
BY THE FLUORESCENT ANTIBODY TEST

ИССЛЕДОВАНИЕ РАЗМНОЖЕНИЯ ВИРУСА VARICELLA-ZOSTER С ПО-
МОЩЬЮ ФЛЮОРЕСЦЕНТНЫХ АНТИТЕЛ

М. Коллер, Е. Гёнциёл, Л. Вац

Исследованы патогистологические изменения в фибробластных культурах чело-веческого эмбриона, вызванные вирусом *Varicella zoster* в различные сроки после зара-жения, сопоставляя их одновременно с выявлением расположенного внутриклеточно вирусного антигена методом иммунофлуоресценции.

Цитопатогенные изменения развиваются приблизительно через 10 часов после заражения, когда в ядрах клеток появляются мелкие эозинофильные глыбки, окружен-ные светлым участком. Спустя 48—72 часа после заражения развиваются характерные ядерные включения типа-А. Гибель клеток наступает через 96—144 часа.

Вирусный антиген могли выявить в первый раз приблизительно на десятом часе после заражения: в ядрах клеток, в форме мелких флуоресцирующих пятен. В 24—48

часов количество антигена в ядрах клеток увеличивается, в то же время цитоплазма заполняется антигеном. Начиная с 72 часов после заражения, вирусный антиген из ядер клеток постепенно исчезает, тогда как специфическая флуоресценция цитоплазмы неизменно сохраняется.

На основании выше изложенных данных можно проводить параллель между внутриклеточным распределением вирусного антигена и появлением ядерных включений типа—А.

K. Incze :

HEAT RESISTANCE OF AEROCOCCUS VIRIDANS (WILLIAMS)

ИССЛЕДОВАНИЕ ТЕРМОУСТОЙЧИВОСТИ AEROCOCCUS VIRIDANS (WILLIAMS)

K. Incze

Определялись данные, характеризующие термоустойчивость *Aerococcus viridans* (значения D и z), сопоставляя с термоустойчивостью вегетативных клеток иных бактерий, и обнаружилось, что термоустойчивость *Aerococcus viridans* значительно выше. Со знанием данных термоустойчивости и проникающей способности тепла, определена потребность в термической обработке презервных товаров. Установлено, что при теперешних условиях необходима двух-трехразовая термическая обработка.

I. Szabó, M. Marton, I. Buti, G. Pártai :

ACTINOMYCES FINLAYI N. SP.

ACTINOMYCES FINLAYI N. SP.

И. Сабо, М. Мартон, И. Буту, Г. Партаи

Actinomyces finlayi n. sp. является в секции VII *Actinomyces* (*Streptomyces*) (Szabó, Marton, 1962) внутри подгруппы создаваемой видами *Actinomyces acrimycini* Gauze 1957, *Streptomyces flaveolus* (Waksman, 1919, Waksman, Henrici, 1948), *Str. albo-griseolus* Benedict и другие 1954 (серия: *Griseoflavus*), а также *Act. griseostramineus* Gauze и другие 1957 (серия: *Viridans*), на основе морфологических и физиологических признаков новым выделенным видом. Типовой штамм *Actinomyces finlayi* n. sp. (холотип: R—I—30) образует в вегетативных мицелиях характерный зеленый эндопигмент, поэтому авторами с целью идентификации его, произведены сравнительные исследования всеми доступными типовыми культурами зеленых актиномицетов. Параллельно с этой работой составлен четкий определитель зеленых актиномицетов, принадлежащих к различным сериям (*Flavovirens*, *Murinus*, *Viridans* и др.).

I. Szabó, E. Vandra :

MYCOBACTERIUM MINETTI (PENSO ET AL. 1952)

BACTERIOLOGICAL AND EPIDEMIOLOGICAL OBSERVATIONS

МИКОБАКТЕРИЙ MINETTI (PENSO 1952)

БАКТЕРИОЛОГИЧЕСКИЕ И ЭПИДЕМИОЛОГИЧЕСКИЕ НАБЛЮДЕНИЯ

И. Сабо, Е. Вандра

Авторами выделены и идентифицированы 21 штамм *M. minetti* (Penso) из человеческих секретов. Идентификация проводилась путем морфологических, био- и цитохимических исследований, а также с помощью определения чувствительности к фагам и опытов, проведенных на животных. На основании данных исследований *M. minetti* считается самостоятельным species, принадлежащим к сапрофитным микобактериям. Патогенность его доказать не представляется возможным. Предполагается, что *M.* обладает функцией, подобной функции почвенных бактерий (деминерализация).

G. Ujváry, M. Gregács, B. Lányi, T. Angyal, S. Vörös, G. Páll :

BEOBSACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN
DES SÄUGLINGS- UND KINDESALTERS

I. UNTERSUCHUNGEN DER ROLLE DER ESCHERICHIA COLI-STÄMME

НАБЛЮДЕНИЯ ПО ЭТИОЛОГИИ ГАСТРОЭНТЕРОКОЛИТОВ ГРУДНОГО И
ДЕТСКОГО ВОЗРАСТА. I. ИССЛЕДОВАНИЕ РОЛИ ШТАММОВ
E. COLI

Г. Уйвари, М. Грегач, Б. Ланьи, Т. Андьал, Ш. Вёрёш, Г. Палл

В течение одного года исследовались ферментативные свойства, антиген «О», чувствительность к антибиотикам, энтеропатогенная роль 967 штаммов *E. coli*, полученных из кала 253 больных энтероколитом и 349 здоровых грудных и детей младшего возраста, а также исследовался патологический процесс энтеральных больных и обрабатывались данные этих исследований.

Исходя из результатов бактериологических исследований, проведенных параллельно с клиническими наблюдениями, обращается внимание на важную роль штаммов *E. coli* в происхождении энтеритов грудных детей 1—6 месячного возраста и указывается на факультативную энтеропатогенную роль штаммов *E. coli* «переходящего» характера, появляющихся периодически в толстой кишке, при гастро-энтероколитах грудного возраста.

G. Ujváry, T. Angyal, S. Vörös, B. Lányi, M. Gregács, G. Páll :

BEOBSACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN
DES SÄUGLINGS- UND KINDESALTERS

II. UNTERSUCHUNG DER ROLLE DER KLEBSIELLA-STÄMME

НАБЛЮДЕНИЯ ПО ЭТИОЛОГИИ ГАСТРОЭНТЕРОКОЛИТОВ ГРУДНОГО И
ДЕТСКОГО ВОЗРАСТА. II. ИССЛЕДОВАНИЕ РОЛИ ШТАММОВ KLEBSIELLA

Г. Уйвари, Т. Андьал, Ш. Вёрёш, Б. Ланьи, М. Грегач, Г. Палл

При клинических наблюдениях детей выделены 304 штамма *Klebsiella*. 37,4% штаммов удалось включить в один из 1—30 типов антигенной схемы *Klebsiella*. Самыми частыми типами явились K:28, K:2, K:14. Чистые культуры выделены у больных с тяжелой (10 случаев), средней (41 случай), легкой (82 случая) формой энтероколита. Тип K:28 вызвал две «домашние» вспышки с небольшим распространением. На основании этих данных факультативная энтеропатогенная роль группы *Klebsiella* является вероятной при гастроэнтероколитах грудного и детского возраста.

A. Lengyel, P. Dán, I. Nász, G. Kulcsár, I. Cserba

INFLUENCE OF TEMPERATURE AND pH ON THE HAEMAGGLUTININATING
ACTIVITY AND HAEMAGGLUTINATION-INHIBITION TEST
OF ADENOVIRUSES

ВЛИЯНИЕ ТЕМПЕРАТУРЫ И pH НА РЕАКЦИИ ГЕМАГГЛЮТИНАЦИИ И
ЗАДЕРЖКУ ГЕМАГГЛЮТИНАЦИИ АДЕНОВИРУСА

А. Лендьел, П. Дан, И. Нас, Г. Кулчар, И. Черба

Исследовано влияние температуры и водородной концентрации на агглютинацию эритроцитов крыс аденовирусом типа 9. Среди пяти температур (+4° С, комнатная температура, воздух 37° С, водяная ванна температуры 37 и 40° С) оптимальными оказались 4° С и комнатная температура. Результаты подтвердились исследованиями, проведенными с двумя другими типами (10 и 13).

Исследовали влияние 7 различных значений pH на реакцию гемагглютинации: 6, 7, 7.5, 8, 8.5, 9, 10. Оптимальными pH в первую очередь явилась 9, а затем 8.5 хотя практически в незначительной степени.

Чувствительность взвеси красных кровяных телец к гемагглютинину при отстаивании не снижается, а незначительно повышается, между значениями pH 7—9.

На реакцию задержки гемагглютинации pH не влияло, при применении одинакового количества вируса.

L. Nyiri :

ÉTUDES DES PROPRIÉTÉS DES ENZYMES PÉNICILLINASE ET PÉNICILLINE ACYLASE À L'OCCASION DE LEUR COEXISTENCE

ИССЛЕДОВАНИЕ СВОЙСТВ СОВМЕСТНО ВСТРЕЧАЕМЫХ ПЕНИЦИЛЛИНАЗЫ И ПЕНИЦИЛЛИН-АЦИЛАЗЫ

Л. Ньери

Один точно еще не определенный штамм *Nocardia* одновременно образует энзимы пенициллиназу и пенициллино-ацилазу. Тогда как оптимальное значение pH пенициллино-ацилазы является 8.7, пенициллиназа того-же происхождения, показала самую высокую активность при pH 5.8. Из-за разницы, наблюдаемой между оптимальными значениями pH, активность энзимы пенициллиназы можно было определить и в присутствии пенициллино-ацилазы. Конститутивная, нативная эндопенициллиназа действовала на бензилпенициллин и на 6-аминопенициллановую кислоту в различной степени. Константы Michaelis были в отношении 6-аминопенициллановой кислоты $3.4 \cdot 10^{-3}$ M, в отношении бензилпенициллина $2.5 \cdot 10^{-5}$ M.

Исследовано влияние различных соединений на пенициллиназу и на пенициллино-ацилазу. Натриевые соли бензойной и сульфанильной кислот оказались способными снижать энзимную активность пенициллиназы без нарушения пенициллино-ацилазы. Подобное действие имел пирогаллол. Фенильно-уксусная кислота и хинин снижали в незначительной степени действие пенициллиназы. Фенильно-уксусная кислота мешала действию ацилазы. Степень задерживающего эффекта явилась функцией концентрации фенильно-уксусной кислоты. Установлено, что формальдегид действует на 6-аминопенициллановую кислоту и на пенициллин химическим путем.

A. Jeney, Jr., G. Szabó :

STUDIES ON THE NUCLEIC ACID CONTENT OF STREPTOMYCES STRAINS

ИССЛЕДОВАНИЕ СОДЕРЖАНИЯ НУКЛЕИНОВЫХ КИСЛОТ В ШТАММАХ STREPTOMYCES GRISEUS

А. Йеней, Г. Сабó

Исследовалось содержание нуклеиновых кислот в 2 штаммах *Streptomyces griseus* с короткой и длинной вегетативной фазой, в различные периоды их роста. Данные исследования освещают одну из существенных причин большего расхождения величин, сообщенных в литературе. Выяснено влияние количества инокулированных микроорганизмов на состав нуклеиновых кислот в штаммах.

B. Serény :

DIFFERENTIATION OF ENTERAL BACTERIA BY THE ALKALINE REACTION

ДИФФЕРЕНЦИРОВКА ЭНТЕРАЛЬНЫХ БАКТЕРИЙ ПУТЕМ РЕАКЦИИ ПОДЩЕЛАЧИВАНИЯ

Б. Шерень

Энтеральные бактерии могут размножаться в выраженно кислотном растворе пептоно-поваренной соли, не содержащей углеводов и другие обычные источники углерода и энергии. Щелочные продукты, которые образуются при их размножении подщелачивают питательную среду, что можно определить по изменению цвета индикатора. В культурах некоторых групп бактерий щелочная реакция появляется быстрее и более постоянно, чем у других микроорганизмов. При соответствующем выборе питательной среды и условий культивирования можно на основе реакции подщелачивания члены семейства *Enterobacteriaceae* разделить на отдельные группы. Для этой цели предлагается применять 5 видов пептонной воды, отличающихся друг от друга только по концентрации водородных ионов (рН 5,4—4,7—4,5—4,3 и 4,1). При помощи этого метода исследовали 1423 энтеральных бактерий и установлено следующее: все члены семейства подщелачивают пептонную воду наименьшей кислотности. На основании активности проявляющейся в остальных пептонных водах, энтеральные бактерии можно разделить на слабо, средне и сильно подщелачивающие микроорганизмы. В первую группу входят *Shigella*, *Proteus*, *Providencia*, во вторую *E. Coli*, *Citrobacter*, *Cloaca*, *S. typhi*, а в третью *Klebsiella*, многочисленные *Salmonella*, *Arizona* допустимо и *Serratia*.

D. Karasszon, L. Bodon :

DEMONSTRATION OF THE SWINE-FEVER VIRUS IN TISSUE CULTURE BY IMMUNOFLOUORESCENCE

ВЫЯВЛЕНИЕ ВИРУСА ЧУМЫ СВИНЕЙ, РАЗМНОЖЕННОГО НА ТКАНЕВОЙ КУЛЬТУРЕ, МЕТОДОМ ИММУНОФЛЮОРЕСЦЕНЦИИ

Д. Карассон, Л. Бодон

Вirus чумы свиней размножали в тканевых культурах эмбриональной свиной почки, а вирусный антиген определяли посредством иммунофлюоресценции. Взвесь клеток ткани почки, обработанная трипсином, разливалась по бутылкам Legroux, на дно которых помещали предметные стекла мелкого размера. Развившуюся тканевую культуру заражали «диким» вирусом чумы свиней, а затем через различные промежутки времени обрабатывали сывороткой кролика, иммунизированной лапинизированным вирусом чумы свиней. После этого насаивали на препараты козьего-кроличьего антиглобулина, меченный флюоресцеинизотиоцианатом. Препарат исследовался под флюоресцентным микроскопом. В препаратах обнаружена интенсивная цитоплазматическая флюоресценция. Соответствующие контрольные препараты не окрашивались.

M. Simon, I. Dömök :

STUDIES ON INTRATYPIC VARIANTS OF ECHOVIRUSES
I. SEPARATION OF HAEMAGGLUTINATING AND NON-HAEMAGGLUTINATING
VIRUS PARTICLES FROM ECHOVIRUS TYPE 6

ИССЛЕДОВАНИЕ ВНУТРИТИПОВЫХ ВАРИАНТОВ ВИРУСА ЭХО
I. ВЫДЕЛЕНИЕ ГЕМАГГЛЮТИНИРУЮЩИХ И НЕ ГЕМАГГЛЮТИНИРУЮЩИХ
ЧАСТИЦ У ТИПА ВИРУСА ЭХО 6

М. Шимон, И. Дёмёк

Из 10 выделенных штаммов вируса *Echo* типа 6 дали положительную реакцию гемагглютинации всего 3. На основе поведения одного гемагглютинирующего штамма сделали вывод, что штамм содержит вирионы двоякого рода: гемагглютинирующие (H^+) и не гемагглютинирующие (H^-). Это предположение подтвердилось исследованиями, направленными на отделение их путем адсорбции к колонке фосфорнокислого кальция и элюирования, а также путем адсорбции к эритроцитам и методом разведения до конечного титра. С колонки фосфорнокислого кальция большинство вирионов H^+ элюировалось при концентрации $M\ 0,2$, а вирионы H^- при концентрации $M\ 0,5$ раствора. Вирионы H^+ адсорбировались эритроцитами, вирионы H^- не адсорбировались. Путем пересева «конечных» разведений гемагглютинирующего штамма и с помощью селекции получили линии H^+ и H^- . Первая оказалась до 14, вторая до 9 пассажа гомогенной. Вирионы H^+ и H^- в отношении некоторых свойств отличаются друг от друга. Размножение вирионов H^+ в тканевых культурах почки обезьяны протекает быстро, достигнутый инфективный титр высокий, цитопатологическое изменение полное, они чувствительны к агаровому ингибитору, тогда как вирионы H^- ведут себя противоположно. Оба вириона различны по антигенной структуре.

На основании этих результатов делаются выводы, что все вариации, сообщенные до сих пор в отношении штаммов вируса *Echo* типа 6, объясняются существованием этих вирионов двоякого рода, что встречаемость вирионов двоякого рода можно считать общим явлением у большинства типов энтеровирусов и этим объясняются наблюдаемые вариации.

J. Szita, G. Hegyessy :

GROUP AND TYPE DISTRIBUTION OF HAEMOLYTIC STREPTOCOCCI
IN HUNGARY DURING THE YEARS 1958—1963

ГРУППОВОЕ И ТИПОВОЕ РАСПРЕДЕЛЕНИЕ ГЕМОЛИТИЧЕСКОГО СТРЕПТОКОККА В ВЕНГРИИ В 1958—63 ГГ.

Й. Сита, Г. Хедьешши

В связи с типизацией 1377 штаммов гемолитического стрептококка, исследованных в течение 1958—1963 гг., оцениваются применяемые методы и результаты полученные при их применении. В 82,4% исследованные штаммы принадлежали к группе «А». Внутри группы «А» выявлением субстанции «М» тип штамма был определен в 48,7%, выявлением субстанции «Т» в 84,7%. В 7,5% штаммов не удалось определить тип ни одним из указанных методов. Что касается превалирующих типов (комплексы 5,11..., 3,13, V_{3264} ; 8,25, Imp_{19} , а также типы 3,12 и 1) полученные данные в большей степени совпадают с чехословацкими результатами Ротта 1960—1961 гг. Существенной разницы в превалирующих типах между отдельными частями Венгрии не обнаружено.

G. Ujváry, B. Lányi, M. Gregács, S. Vörös, T. Angyal, G. Páll :

BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

III. UNTERSUCHUNGEN DER ROLLE DER PROTEUS VULGARIS- UND DER PROTEUS MIRABILIS-STÄMME

НАБЛЮДЕНИЯ ПО ЭТИОЛОГИИ ГАСТРОЭНТЕРОКОЛИТОВ ГРУДНОГО И ДЕТСКОГО ВОЗРАСТА. III. ИССЛЕДОВАНИЯ РОЛИ ШТАММОВ PROTEUS VULGARIS, PROTEUS MIRABILIS

Г. Уйвари, Б. Ланьи, М. Грегач, Ш. Вёрёш, Т. Андьал, Г. Палл

Авторами исследованы дети грудного и детского возраста, 376 с энтеральными и 638 с экстраэнтеральными заболеваниями, с целью выяснения энтеро-патогенной роли штаммов *Proteus vulgaris* и *mirabilis*. Результаты исследований кала показали, что значительная часть детей, бывших в больнице, без учета патологического процесса, — имеет своеобразную «больничную» флору *Proteus*, которая существенно отличается от флоры здоровых и характеризуется высокой распространенностью штаммов, принадлежащих к группам 0:3, 0:6, 0:26 и 0:28 *P. mirabilis*.

При исследовании 376 энтеральных больных, выделены из кала 98 больных *P. vulgaris*, *P. mirabilis*. Штаммы *P. vulgaris*, *P. mirabilis* в 36 случаях выделены вместе с *E. coli dyspepsiae* или с другими хорошо известными энтеральными патогенными бактериями. На основании клинической оценки 62 «чистых случаев *proteus*» с вероятностью можно предполагать факультативную энтеропатогенную роль штаммов *Proteus*.

G. Ujváry, S. Vörös, T. Angyal, B. Lányi, M. Gregács, G. Páll :

BEOBACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN DES SÄUGLINGS- UND KINDESALTERS

IV. UNTERSUCHUNG DER ROLLE DER PROTEUS MORGANII-STÄMME

НАБЛЮДЕНИЯ ПО ЭТИОЛОГИИ ГАСТРОЭНТЕРОКОЛИТОВ У ДЕТЕЙ ГРУДНОГО И ДЕТСКОГО ВОЗРАСТА. IV. ИССЛЕДОВАНИЯ РОЛИ ШТАММОВ PROTEUS MORGANII

Г. Уйвари, Ш. Вёрёш, Т. Андьал, Б. Ланьи, М. Грегач, Г. Палл

Из кала 240 детей грудного и детского возраста выделены 257 штаммов *P. morganii*. 82% штаммов, при помощи 28 групповых сывороток «0», смогли включить в какую-либо группу «0». Обнаружено 11 различных антигенов «0» (78%). В значительном количестве выделены группы 02, 04, 016.

В отношении распределения по серологическим группам штаммов, выделенных от энтеральных больных и контрольных лиц, а также, что касается количества неопределяемых в этих двух группах штаммов, разницы не обнаружено.

При исследовании чувствительности к антибиотикам бросается в глаза высокая толерантность штаммов к антибиотикам. Среди последних наиболее эффективным оказался неомисин (99%).

На основании исследований авторы считают этиологическую роль штаммов *P. morganii* при энтероколитах детского возраста мало вероятной.

G. Ujváry, T. Angyal, B. Lányi, S. Vörös, M. Gregács, G. Páll :

BEOBSACHTUNGEN ÜBER DIE ÄTIOLOGIE DER GASTROENTEROCOLITIDEN
DES SÄUGLINGS- UND KINDESALTERS

V. UNTERSUCHUNG DER ROLLE DER PSEUDOMONAS AERUGINOSA-
UND DER STAPHYLOCOCCUS AUREUS-STÄMME

НАБЛЮДЕНИЯ ПО ЭТИОЛОГИИ ГАСТРОЭНТЕРОКОЛИТОВ У ДЕТЕЙ ГРУДНОГО
И ДЕТСКОГО ВОЗРАСТА. V. ИССЛЕДОВАНИЯ РОЛИ ШТАММОВ
PSEUDOMONAS AERUGINOSA, STAPHYLOCOCCUS AUREUS

Г. Уйвари, Т. Андьал, Б. Ланьи, Ш. Вёрёш, М. Грегач, Г. Палл

1. Сообщаются результаты исследований по энтеропатогенной роли 32 штаммов *Pseudomonas aeruginosa* и 25 штаммов *Staphylococcus aureus*, взятых из кала детей грудного и детского возраста с энтеральными (376) и экстраэнтеральными (638) заболеваниями.

2. Обе разновидности из кала больных с энтеритом выделялись несколько чаще чем у больных не страдающих энтеральными заболеваниями.

3. В кале относительно чаще встречались штаммы *Ps. aeruginosa* принадлежащие к серологическим группам 0:4, 0:5. Среди штаммов *Staphylococcus*, происходящих от экстраэнтеральных патологических процессов, включили в 4 серологическую группу, а большинство штаммов, выделенных из кала во 2. серологическую группу.

4. Что касается чувствительности к антибиотикам, штаммы *Staphylococcus*, происходящие из кала, особенно принадлежащие ко второй серологической группе, отличались полирезистентностью, а штаммы, выделенные от экстраэнтеральных, пиогенных процессов, в большинстве случаев принадлежащие к 4 серологической группе, отличались поличувствительностью.

5. Энтериты, приписываемые штаммам *Ps. aeruginosa*, могут встречаться у детей грудного возраста, а энтериты стафилококкового происхождения в одинаковой степени встречаются как у детей грудного возраста, так и более старшего возраста.

6. При лечении антибиотиками отмечается отчетливое клиническое улучшение и прекращение выделения бактерий.

7. На основании исследования энтеральных больных, по отдельности, по анализу клинических и лабораторных данных, можно считать возможной энтеропатогенную роль штаммов *Ps. aeruginosa*, *Staphylococcus aureus* в гастроэнтероколитах грудного и детского возраста.

Horváth J. :

DOMINANCE IN HYBRIDS DERIVED FROM CROSSING OF STREPTOMYCETES

ВОПРОСЫ ДОМИНАЦИИ У ПОТОМКОВ,
ВОЗНИКАЮЩИХ ПРИ ПЕРЕКРЕЩИВАНИИ STREPTOMICETES

Й. Хорват

Автор произвел перекрещивание *S. rimosus* с *S. griseus* не образующим стрептомицин, а также с *S. globisporus* имеющим широкий антибиотический спектр действия. У гибридов появилась доминанция важнейших признаков переданного свойства одной из родительских клеток. Доминирующими явились *S. griseus*, *S. globisporus*, рецессивным *S. rimosus*. Доминирующими свойствами оказались образование и форма воздушных мицелий, их способность разлагать желатину и антибиотическое действие. Не являлись доминирующими и отличались от родительских клеток форма клеток и количество образованного антибиотика. Образование последнего почти прекратилось у гибридов видов с высокой продукцией. Доминанция гибридов обнаруживается только в случае, когда перекрещиваются виды доминантного и рецессивного характера в отношении упомянутых маркеров. При перекрещивании рецессивных видов возникают крайне изменчивые гибридные потомки.

I. Nász :

SOME BIOLOGICAL CHARACTERISTICS OF ADENOVIRUS TYPE 8 STRAINS

НЕКОТОРЫЕ БИОЛОГИЧЕСКИЕ СВОЙСТВА ШТАММОВ АДЕНОВИРУСА ТИПА 8

И. Нас

Автором изучались 13 штаммов аденовируса, принадлежащие к типу 8. Два штамма были лабораторными, 10 выделены от больных с кератоконъюнктивитом. Все штаммы без исключения вызвали «дегенерацию третьего типа» на первичных тканевых культурах амниона человека.

Цитопатогенное влияние штаммов при применении одинакового количества вируса на более старых тканевых культурах (30 дневные или старше) появлялось ранее и более отчетливо, чем в более молодых (5—10 дневные культуры).

Количество вируса на питательной жидкости тканевых культур было низким (100—300 TCID₅₀/мл).

Оптимальная адсорбция к клеткам наступает через 300 минут. При применении оптимального адсорбционного времени и заражении дозами вируса 1, 2, 4, 8, 20 и 40 TCID₅₀/мл. в темпе развития укомплектования дегенерации тканевых культур могли различать 3 зоны. Пропорционально уменьшению вирусных доз было необходимо больше времени для развития 25% прогрессирования дегенерации. Может быть этим явлением объясняется, что выделение аденовирусных штаммов типа 8 является часто тяжелым и длительным процессом.

E. Molnár :

A SEROLOGICAL STUDY OF THE INCIDENCE OF TICK-BORNE ENCEPHALITIS IN HUNGARY

РАСПРОСТРАНЕННОСТЬ КЛЕЩЕВОГО ЭНЦЕФАЛИТА В ВЕНГРИИ НА ОСНОВАНИИ СЕРОЛОГИЧЕСКИХ ИССЛЕДОВАНИЙ

Е. Молнар

В связи с 840 случаями различных заболеваний нервной системы, автор с 1958 г. до конца 1963 г. исследовал 1230 проб крови на реакцию нейтрализации вируса клещевого энцефалита. Среди 280 больных с энцефалитом 25% оказались положительными, среди 459 больных с асептическим менингитом 13%, тогда как среди 91 больного с подозрением на полиомиелит ни один не дал положительную реакцию. Частота встречаемости антител повышалась параллельно возрасту.

Автор обнаружил почти на всех административных территориях Венгрии случаи клещевого энцефалита, которые подтвердились при серологических исследованиях. Значительная часть заболеваний протекала в виде асептического менингита.

I. Gadó, I. Horváth :

THE EFFECT OF METHANOL ON THE GROWTH OF STREPTOMYCIN-DEPENDENT STRAINS OF *E. COLI* IN STREPTOMYCIN FREE MEDIA

ВЛИЯНИЕ МЕТАНОЛА НА РАЗМНОЖЕНИЕ ШТАММОВ *E. COLI* STREPTOMYCIN DEPENDENT НА ПИТАТЕЛЬНОЙ СРЕДЕ, НЕ СОДЕРЖАЩЕЙ СТРЕПТОМИЦИН

И. Гадо, И. Хорват

Известно, что при инокуляции штамма *E. coli streptomycin dependent* на питательной среде, не содержащей стрептомицин, возникают удлиненные формы, рост которых задерживается метанолом и некоторыми другими органическими растворителями, а также сахарозой. При таких условиях и в случае отсутствия стрептомицина обнаруживается размножение, интенсивность которого зависит от питательной среды. Результаты указывают на то, что у штаммов *streptomycin dependent* стрептомицин играет роль в синтезе клеточной оболочки и в клеточном делении.

I. Nász, A. Lengyel, P. Dán, G. Kulcsár :

HETEROTYPIC HAEMAGGLUTINATION INHIBITION IN THE ADENOVIRUS GROUP

РЕАКЦИЯ ГЕТЕРОТИПИЧНОЙ ЗАДЕРЖКИ ГЕМАГГЛЮТИНАЦИИ МЕЖДУ ОТДЕЛЬНЫМИ ТИПАМИ АДЕНОВИРУСА

И. Нас, А. Лендьел, П. Дан, Г. Кулчар

Двукратно исследовались сыворотки 40 больных с эпидемическим кератоконъюнктивитом, а также 208 проб человеческой крови на содержание антител задерживающих гемагглютинацию в отношении типов 8, 9, 10 аденовируса. Обнаружили гетеротипичные реакции между этими тремя штаммами. Соответствующие результаты дали перекрестные реакции гемагглютинации, проведенные типовыми сыворотками кроликов.

T. Szabados, J. Böszörményi, G. Dobiás, M. Rojti, V. P. Juhász :

EFFECT OF IODOCASEIN FEEDING ON THE ANTITOXIN TITRE OF ANIMALS USED IN SERUM PRODUCTION

ЭФФЕКТ КОРМЛЕНИЯ ИОД-КАЗЕИНОМ НА ТИТР АНТИТОКСИНА ЖИВОТНЫХ, ИСПОЛЬЗОВАННЫХ ДЛЯ ИЗГОТОВЛЕНИЯ СЫВОРОТОК

Т. Сабадош, И. Бёсёрменьи, Г. Добиаш, М. Ројти, В. П. Юхас

1. Титр антитоксина лошади, иммунизированной дифтерийным антигеном в течение 21 месяца непрерывно, под влиянием кормления иод-казеином, повышается через 2 месяца примерно втрое.

2. Из 10 овец, иммунизированных стафилококковым токсином, титр антитоксина у 4 животных, подкармливаемых иод-казеином, в течение всего эксперимента, продолжавшегося 13 месяцев, был выше, чем у 6 контрольных животных. Максимальная разница в титрах была 256%.

3. У 22 животных крупного рогатого скота, иммунизированных столбнячным антигеном, не обнаружилось однозначное расхождение между антитоксическими уровнями подкармливаемых иод-казеином и контрольных животных.

4. Из 20 лошадей, иммунизированных в течение 6 месяцев столбнячным антигеном, титр антитоксина 10 животных, получавших иод-казеин, остались до конца выше, чем

титры контрольной группы, но разница была только в короткий промежуток времени достоверной. Максимальное расхождение между средними титрами двух групп была 45%.

5. Повышение антитоксического уровня обнаруживается после дачи иод-казеина в течение 6—8 недель.

6. Усиливающее действие, в отношении образования антител, иод-казеина изменялось по видам животных, наиболее выраженным было оно у овец, менее выраженным у лошадей, тогда как у крупного рогатого скота не обнаруживалось однозначное действие.

7. Усиливающее действие в отношении продукции антител иодказеина при введении стафилококкового, дифтерийного и столбнячного токсинов проявилось в одинаковой степени.

8. Эти результаты подтверждают данные тех авторов, которые с помощью средств, усиливающих обмен веществ, могли повышать образование антител и до некоторой степени подтверждают «конвейерную» теорию Гунтера. Усиление обмена веществ практически применяется при производстве сывороток, для повышения титра антител животных-продуцентов.

L. Váci, Gy. Hadházy, É. Horváth :

THE INFLUENCE OF TEMPERATURE ON THE MULTIPLICATION
OF THE PR8 STRAIN OF INFLUENZA A VIRUS
AND ON THE INTERFERON PRODUCTION BY THE VIRUS-INFECTED CELLS
ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА РАЗМНОЖЕНИЕ ШТАММА PR8 ВИРУСА
ГРИППА «А» И НА ОБРАЗОВАНИЕ ИНТЕРФЕРОНА КЛЕТКАМИ, ЗАРАЖЕН-
НЫМИ ВИРУСОМ

Л. Вац, Дь. Хадхази, Е. Херват

На курином эмбрионе исследовалось размножение штамма PR8 и образование интерферона при различных температурах.

Размножение вируса при 39 и 40° С в течение 12 часов было быстрее, чем при 35° С. При температурах 41 и 42° С в этот период снижалась интенсивность размножения вируса. Самые высокие титры были обнаружены к концу 24 часов, независимо от температуры инкубации. Продуктивность была оптимальной при температуре 35° С, а при температуре 39 и 42° С, параллельно с повышением температуры, было обнаружено снижение продуцируемого количества вируса.

При температуре 35° С образование интерферона смогли выявить первый в раз на 24 часу и начиная с этого времени титр интерферона повышался до 96 часов. Первоначальное образование интерферона было повышенным при температуре 39 и 41° С, но при температурах 40 и 41° С после 24 часов образование его прекратилось. Сопоставляя титры, полученные при температуре 35° С, при температуре 39,40 и 41° С на 24 часу обнаружили 2,4 и 8 кратный титр.

На куриных эмбрионах, которые находились в инкубации в течение 24 часов при 41° С и при 35° С, продукция интерферона не прекратилась на 24 часу.

B. Serény :

BREAKDOWN OF AMINO-ACIDS BY ENTEROBACTERIACEAE:
ITS USE AS A ROUTINE DIAGNOSTIC TEST

ДЕЗАМИНАЦИЯ ЭНТЕРАЛЬНЫХ БАКТЕРИЙ НА ПИТАТЕЛЬНЫХ
СРЕДАХ, СОДЕРЖАЩИХ АМИНОКИСЛОТЫ. ИССЛЕДОВАНИЯ
ДЛЯ ПРИМЕНЕНИЯ РЕАКЦИИ В КАЧЕСТВЕ ЕЖЕДНЕВНОЙ
ДИАГНОСТИЧЕСКОЙ ПРОБЫ

Б. Шерень

Автором составлена синтетическая среда, пригодная для ежедневного исследования активности дезаминазы энтеральных бактерий. На основании еще относительно не многочисленных исследований автор предлагает ввести данный метод для более широкого ежедневного применения для дифференциальной диагностики энтеральных бактерий.

G. Ivánovics, I. Varga, E. Marjai :

AUXOTROPHS OF BACILLUS ANTHRACIS

АУКСОТРОФЫ BACILLUS ANTHRACIS

Г. Иванович, И. Варга, Е. Марjai

Применяя акапсулогенный мутант штамма *Bacillus anthracis Vollum* в качестве штамма «parent» изолировали 186 мутантов, которые обнаружили более высокую потребность к питательным веществам, чем штамм «parent». В то время, как штамм «parent» при наличии смеси аминокислот (гидролизат кислого казеина + триптофан и цистин) — а также тиамина, при температуре 37 °C культивировался субоптимально, мутанты показали такой же рост только в присутствии добавочных метаболитов. Эти мутанты с повышенной потребностью считаются ауксотрофными *B. anthracis*. Из 186 изолированных мутантов 99 оказались с повышенной потребностью к пуринам, а 57 к пиримидину. Остальные 25 штаммов имели специфическую потребность к различным витаминам (биотин, ПАВА, амид никотиновой кислоты, рибофлавин). Повышенную потребность остальных 5-ти мутантов при проведенных исследованиях не удалось установить. Больше половины штаммов с повышенной потребностью к тиамину или биотину проявили это свойство только при температуре 37 °C, тогда как при температуре 26 °C вели себя как штаммы «parent».

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